REMARKS

Applicants thank the Examiner for the review of the instant application. Claims 42 and 43 have been cancelled; Claim 47 has been amended to better define the extracellular domain, and Claim 50 has been amended to depend from Claim 44 rather than cancelled Claim 42. Thus, Claims 44-45, 47, and 49-55 are presented for further examination. For the reasons stated below, Applicants respectfully traverse the rejection of the pending claims.

Rejection Under 35 U.S.C. §101

The PTO maintains its rejection of Claims 44-45, 47, and 49-55 under 35 U.S.C. § 101 as lacking a substantial asserted utility or a well-established utility for the reasons set forth in the previous Office Actions. The PTO asserts that one skilled in the art would not know how to use the claimed invention. According to the PTO, "[t]he data set forth in the specification are preliminary at best because the specification does not teach the expression of the PRO180 polypeptide nor any particular biological activity of the polypeptide." *Final Office Action* at 3. The PTO relies on Hu *et al.* and other references for the propositions that the literature cautions researchers against drawing conclusions based on small changes in transcript expression levels between normal and cancerous tissue, and that what is often seen is a lack of correlation between DNA expression and increased peptide levels. The PTO argues that further research is required to determine whether the PRO180 polypeptide is differentially expressed, making the asserted utility for the claimed polypeptides not substantial.

Applicants have previously set forth the legal standard for utility. It is established that the legal standard for demonstrating utility is a relatively low hurdle. An Applicant need only provide evidence such that it is **more likely than not that a person of skill in the art would be convinced, to a reasonable probability, that the asserted utility is true**. The evidence need not be direct evidence, so long as there is a reasonable correlation between the evidence and the asserted utility. The Applicant **does not need to provide evidence such that it establishes an asserted utility as a matter of statistical certainty.**

Even assuming that the PTO has met its initial burden to offer evidence that one of ordinary skill in the art would reasonably doubt the truth of the asserted utility, Applicants assert

that they have met their burden of providing rebuttal evidence such that it is more likely than not those skilled in the art, to a reasonable probability, would believe that the claimed invention is useful as a diagnostic tool for cancer.

Substantial Utility

Summary of Applicants' Arguments and the PTO's Response

Applicants first offer a summary of their argument and the disputed issues involved. Applicants assert that the claimed polypeptides have utility as diagnostic tools for cancer, particularly lung and rectum tumors. Applicants' asserted utility rests on the following argument:

1. Applicants have provided reliable evidence that mRNA for the PRO180 polypeptide is expressed at least two-fold higher in rectal tumor tissue and in normal lung tissue compared to normal rectal tissue and lung tumor tissue;

2. Applicants assert that it is well-established in the art that a change in the level of mRNA for a particular protein, e.g. an increase, generally leads to a corresponding change in the level of the encoded protein, e.g. an increase;

3. Given Applicants' evidence that the mRNA for the PRO180 polypeptide is differentially expressed in rectal and lung tumor tissue, it is more likely than not that the PRO180 polypeptide is likewise differentially expressed in these tumors; the claimed polypeptides are therefore useful as diagnostic tools to distinguish rectal tumor tissue from normal rectal tissue, and lung tumor tissue from normal lung tissue.

Applicants understand the PTO to be asserting that "[t]he data set forth in the specification are preliminary at best because the specification does not teach the expression of the PRO180 polypeptide nor any particular biological activity of the polypeptide" and that "the skilled artisan would need to perform additional experiments to reasonably confirm" that the PRO180 polypeptide is differentially expressed in rectal and lung tumor.

As detailed below, Applicants submit that the PTO has failed to demonstrate that this is one of the "rare cases" where the applicants have "asserted a utility that could only be true if it violated a scientific principle, such as the second law of thermodynamics, or a law of nature, or was wholly inconsistent with contemporary knowledge in the art." *M.P.E.P.* § 2107.02 III B.

The references cited by the PTO in support of its rejections are either irrelevant, not contrary to Applicants' arguments, or actually offer support for Applicants' position. Even if the PTO has met its initial burden, Applicants have submitted enough rebuttal evidence such that it is more likely than not that a person of skill in the art would be convinced, to a reasonable probability, that the asserted utility is true. As stated above, Applicants' evidence need not be direct evidence, so long as there is a reasonable correlation between the evidence and the asserted utility. The standard is not absolute certainty.

Applicants have established that the Gene Encoding the PRO180 Polypeptide is Differentially Expressed in Certain Cancers compared to Normal Tissue

Applicants first remind the PTO of the level of evidence required to support a substantial utility.

> [T]he Appellant does not have to provide evidence sufficient to establish that an asserted utility is true "beyond a reasonable doubt." Nor must the Appellant provide evidence such that it establishes an asserted utility as a matter of statistical certainty. Instead, evidence will be sufficient if, considered as a whole, it leads a person of ordinary skill in the art to conclude that the asserted utility is more likely than not true. M.P.E.P. at § 2107.02, part VII (emphasis in original, citations omitted).

The Court of Appeals for the Federal Circuit has stated that the standard for satisfying the utility requirement is a low one:

> The threshold of utility is not high: An invention is "useful" under section 101 if it is capable of providing some identifiable benefit. *See Brenner v. Manson,* 383 U.S. 519, 534, 86 S.Ct. 1033, 16 L.Ed.2d 69 (1966); *Brooktree Corp. v. Advanced Micro Devices, Inc.,* 977 F.2d 1555, 1571 (Fed. Cir. 1992) ("To violate § 101 the claimed device must be totally incapable of achieving a useful result"); *Fuller v. Berger,* 120 F. 274, 275 (7th Cir.1903) (test for utility is whether invention "is incapable of serving any beneficial end"). *Juicy Whip, Inc. v. Orange Bang, Inc.*, 185 F.3d 1364, 1366, 51 U.S.P.Q. 2d 1700 (Fed. Cir. 1999) (emphasis added).

The low threshold for satisfying the utility requirement is reflected in the standard set by the Federal Circuit for invalidating a patent based on a lack of utility: "[T]he fact that an invention has only limited utility and is only operable in certain applications is not grounds for finding lack of utility. Some degree of utility is sufficient for patentability. Further, the defense of non-utility

cannot be sustained without proof of total incapacity." *Envirotech Corp. v. Al George, Inc.*, 730 F.2d 753, 762, 221 U.S.P.Q. 473 (Fed. Cir. 1984) (emphasis added, citations omitted).

Because the standard for satisfying the utility requirement is so low, requiring total incapacity for a finding of no utility, the M.P.E.P. cautions that:

> Rejections under 35 U.S.C. 101 have been *rarely* sustained by federal courts. Generally speaking, in these *rare* cases, the 35 U.S.C. 101 rejection was sustained [] because the Appellant ... asserted a utility that could only be true if it violated a scientific principle, such as the second law of thermodynamics, or a law of nature, or was wholly inconsistent with contemporary knowledge in the art. M.P.E.P. § 2107.02 III B., citing *In re Gazave*, 379 F.2d 973, 978, 154 U.S.P.Q. 92, 96 (C.C.P.A. 1967) (underline emphasis in original, italic emphasis added).

In *Nelson v. Bowler*, 626 F.2d 853, 206 U.S.P.Q. 881 (C.C.P.A. 1980), the court held that crude screens for pharmacological activity which were reported as qualitative results without statistical analysis were sufficient to establish utility. The Appellants in *Nelson* relied on two tests to prove practical utility for derivatives of naturally occurring prostaglandins: an *in vivo* rat blood pressure (BP) test and an *in vitro* gerbil colon smooth muscle stimulation (GC-SMS) test. In the BP test, responses to the compounds were categorized qualitatively, as either a depressor (lowering) effect or a pressor (elevating) effect. *Nelson*, 626 F.2d at 854-55. In the GC-SMS test a section of colon was excised from a freshly-killed gerbil for suspension in a physiological solution, and a lever arm was connected to the colon in such a way that any contraction was recorded as a polygraph trace. *Id.* The Board held that Nelson had not shown adequate proof of practical utility, characterizing the tests as "rough screens, uncorrelated with actual utility." *Id.* at 856.

On appeal the C.C.P.A. reversed, holding that the Board "erred in not recognizing that tests evidencing pharmacological activity may manifest a practical utility even though they may not establish a specific therapeutic use." *Id.* (emphasis added). The Court stated that "practical utility" was characterized as a use of the claimed discovery in a manner which provides some immediate benefit to the public, establishing the rule that "[k]nowledge of the pharmacological activity of any compound is obviously beneficial to the public.... [W]e conclude that adequate proof of any such activity constitutes a showing of practical utility." *Id.* (emphasis added).

The Court rejected Bowler's argument that the BP and GC-SMS tests are inconclusive showings of pharmacological activity since confirmation by statistically significant means did not

occur until after the critical date. The Court stated that "a rigorous correlation is not necessary where the test for pharmacological activity is reasonably indicative of the desired response." *Id.* (emphasis added). The Court concluded that a "reasonable correlation" between the observed properties and the suggested use was sufficient to establish practical utility. *Id.* at 857.

The test articulated in *Nelson* is certainly met by the evidence in Example 18. Presented with the data in Example 18, one of skill in the art would find that there is a "reasonable correlation" between the observed property of differential expression in certain tumors and the suggested use as a diagnostic tool for cancer. In *Nelson* the fact that the results were qualitative, not statistically significant, and preformed *in vivo* in rats or *in vitro* on gerbil colon did not matter. The Court held that statistically significant results are not required, nor is it necessary to prove actual clinical therapeutic usefulness.

The gene expression data in the specification, Example 18, shows that the mRNA associated with the PRO180 polypeptide was more highly expressed in rectal tumor tissue than in normal rectal tissue, and in normal lung tissue compared to lung tumor tissue. Gene expression was analyzed using standard semi-quantitative PCR amplification reactions of cDNA libraries isolated from different human tumor and normal human tissue samples. Identification of the differential expression of the PRO180 polypeptide-encoding gene in tumor tissue compared to the corresponding normal tissue renders the molecule useful as a diagnostic tool for the determination of the presence or absence of tumor. Applicants previously submitted a first Declaration of J. Christopher Grimaldi, an expert in the field of cancer biology. This declaration explains the importance of the data in Example 18, and how differential gene and protein expression studies are used to differentiate between normal and tumor tissue (see Declaration, paragraph 7).

With respect to the PTO's concerns regarding the data in Example 18, Applicants maintain that the methodology used to compare mRNA levels in normal tissue to that in cancerous tissue is reliable. In paragraphs 6 and 7, Mr. Grimaldi explains that the semi-quantitative analysis employed to generate the data of Example 18 is sufficient to determine if a gene is over- or under-expressed in tumor cells compared to corresponding normal tissue. He states that any visually detectable difference seen between two samples is indicative of at least a two-fold difference in cDNA between the tumor tissue and the counterpart normal tissue. Thus,

the results of Example 18 reflect at least a two-fold difference between normal and tumor samples. He also states that the results of the gene expression studies indicate that the genes of interest "can be used to differentiate tumor from normal," thus establishing their reliability. He explains that, "The precise levels of gene expression are irrelevant; what matters is that there is a relative difference in expression between normal tissue and tumor tissue." (Paragraph 7). Thus, since it is the relative level of expression between normal tissue and suspected cancerous tissue that is important, the precise level of expression in normal tissue is irrelevant. Likewise, there is no need for quantitative data to compare the level of expression in normal and tumor tissue. As Mr. Grimaldi states, "If a difference is detected, this indicates that the gene and its corresponding polypeptide and antibodies against the polypeptide are useful for diagnostic purposes, to screen samples to differentiate between normal and tumor."

The PTO rejects the Grimaldi declaration on various grounds, including that "Mr. Grimaldi is named as one of the inventor [*sic*] and is employed by the assignee." *Final Office Action* at 9. Applicants note that an affidavit cannot be disregarded solely because it is signed by the applicant. (See M.P.E.P. §716.01(c)). Furthermore, Applicants maintain that Mr. Grimaldi's first Declaration objectively sets forth the methodology employed in the experiments described in Example 18 and the conclusions derived therefrom.

In sum, the data in Example 18 are sufficient to establish a practical utility for the claimed invention. Applicants are asserting that the PRO180 gene, polypeptide and antibodies have utility as diagnostic tools for cancer, particularly rectal and lung tumor. Applicants are not asserting that the PRO180 gene, polypeptide and antibodies necessarily provide a definitive diagnosis of cancer, but rather that they are useful, alone or in combination with other diagnostic tools, to assist in the diagnosis of rectal and lung tumor. Statistically significant results are not required, nor is it necessary to prove actual clinical therapeutic usefulness.

Further, Applicants submit that a lack of known role for PRO180 in cancer does not prevent its use as a diagnostic tool for cancer. There is a difference between use of a gene for distinguishing between tumor and normal tissue on the one hand, and establishing a role for the gene in cancer on the other. Genes with lower levels of change in expression may or may not be the most important genes in causing the disease, but the genes can still show a consistent and measurable change in expression. While such genes may or may not be good targets for further

research, they can nonetheless be used as diagnostic tools. The PRO180 gene can be used as a cancer diagnostic tool because it is differentially expressed in rectal and lung tumors.

While it is true that the specification provides only mRNA expression data, as Applicants explain in detail below, one of skill in the art would accept that increases or decreases in mRNA level for a particular gene are reasonably correlated with increases or decreases in the encoded polypeptide level, respectively. Therefore, there is a clear nexus between the differential expression of PRO180 mRNA in lung and rectal tumors and the differential expression of the PRO180 polypeptide in these same tissue types.

Hu et al. and LaBaer

Applicants next turn to the PTO's arguments based on Hu *et al.* and LaBaer. As previously pointed out, Hu et al. used an automated literature-mining tool to summarize and estimate the relative strengths of all human gene-disease relationships published on Medline. They then generated a microarray expression dataset comparing breast cancer and normal breast tissue. Using their data-mining tool, they looked for a correlation between the strength of the literature association between the gene and breast cancer, and the magnitude of the difference in expression level. They report that for genes displaying a 5-fold change or less in tumors compared to normal, there was no evidence of a correlation between altered gene expression and a known role in the disease. *See* Hu at 411. However, among genes with a 10-fold or more change in expression level, there was a strong correlation between expression level and a published role in the disease. *Id.* at 412. Importantly, Hu reports that the observed correlation was only found among estrogen receptor-positive tumors, not ER-negative tumors. *Id.*

The general findings of Hu are not surprising – one would expect that genes with the greatest change in expression in a disease would be the first targets of research, and therefore have the strongest known relationship to the disease as measured by the number of publications reporting a connection with the disease. The correlation reported in Hu only indicates that the greater the change in expression level, the more likely it is that there is a published or known role for the gene in the disease, as found by their automated literature-mining software. Thus, Hu's results merely reflect a bias in the literature toward studying the most prominent targets, and reflect nothing regarding the ability of a gene that is 2-fold or more differentially expressed in tumors to serve as a disease marker.

More importantly, Hu did not look for a correlation between changes in mRNA and changes in protein levels, and therefore is not contrary to Applicants' assertion that there is a correlation between the two. Applicants are not relying on any "role" that PRO180 has in cancer for their asserted utility. Instead, Applicants are relying on the differential expression of PRO180 in certain tumors compared to their normal tissue counterparts. Nowhere in Hu does it say that a lack of correlation in their study means that genes with a less than five-fold change in level of expression in cancer cannot serve as a molecular marker of cancer.

The PTO also relies on the publication by LaBaer to broaden its interpretation of Hu. *Final Office Action* at 5. The PTO points to a statement in LaBaer that "reports of mRNA or protein changes of as little as two-fold are not uncommon, and although changes of this magnitude may turn out to be important, most are attributable to disease-independent differences between samples." *Final Office Action* at 5, citing *LaBaer* at 976.

LaBaer is an unreviewed letter to the editor by an author of the Hu *et al.* article describing the automated literature searching tool used in the Hu *et al.* reference discussed above. LaBaer provides no further evidence than that provided in Hu, and provides no evidence whatsoever to support the conclusion that the results of Hu are applicable to the diagnostic utility of differentially expressed genes. Importantly, like the Hu reference, LaBaer does not consider or offer any discussion of whether there is a correlation between changes in mRNA levels and changes in the level of the encoded protein.

In addition, it is important to note that Applicants' are not relying on microarray data as discussed in Hu and LaBaer, as well as in Hanash et al., Winstead, and Irving et al., all cited by the PTO on page 14 of the final Office Action. Instead, Applicants are relying on a more accurate and reliable method of assessing changes in mRNA level, namely quantitative PCR analysis. In a recent study by Kuo *et al.*, (Proteomics 5(4):894-906 (2005)), the authors used microarray analysis combined with proteomic analysis using two-dimensional gel electrophoresis to examine changes in gene expression in leukemia cell lines, just as discussed in LaBaer. The authors report that "[c]omparison of microarray and proteomic expression profiles showed poor correlation. Use of more reliable and sensitive analyses, such as reverse transcriptase polymerase chain reaction [RT-PCR], Western blotting and functional assays, on several genes and proteins, nonetheless, confirmed that there is indeed good correlation between mRNA and protein

expression." Kuo *et al.* at Abstract (emphasis added) (attached as Exhibit 1). Thus, even if accurate, the statements in Hu, LaBaer, Winstead and Irving et al. regarding microarray studies are not relevant to the instant application which does not rely on microarray data.

Moreover, LaBaer is silent regarding the reliability of pooled samples, and whether or not differential expression in pooled samples are susceptible to disease-independent differences between samples. LaBaer's conclusions regarding disease-independent differences between samples are not applicable in the instant case where normal human tissue and corresponding human tumor tissue samples were used. Accordingly, LaBaer suffers from the same defects discussed above with respect to Hu *et al.*

Nothing in either LaBaer or Hu or Winstead or Irving addresses Applicants' assertion that there is a correlation between changes in mRNA level and changes in the level of the encoded polypeptide. As such, they are not relevant to establishing whether the claimed polypeptides have utility based on the differential expression of the PRO180 mRNA in rectal and lung tumors.

In conclusion, Applicants submit that the evidence reported in Example 18, supported by the first Grimaldi Declaration, establish that there is at least a two-fold difference in PRO180 cDNA between rectal and lung tumors and normal rectal and lung tissue, respectively. Therefore, it follows that expression levels of the PRO180 gene can be used to distinguish rectal tumor tissue from normal rectal tissue, and lung tumor tissue from normal lung tissue. As Applicants explain below, it is more likely than not that the PRO180 polypeptide can also be used to distinguish rectal tumor tissue from normal rectal tissue, and lung tumor tissue from normal lung tissue.

Applicants have established that the Accepted Understanding in the Art is that there is a Positive Correlation between Changes in mRNA Levels and Changes in the Level of Expression of the Encoded Protein

Applicants next turn to the second portion of their argument in support of their asserted utility – that it is well-established in the art that a change in the level of mRNA for a particular protein, generally leads to a corresponding change in the level of the encoded protein; given Applicants' evidence of differential expression of the mRNA for the PRO180 polypeptide in rectal and lung tumors, it is likely that the PRO180 polypeptide is likewise differentially

expressed in these tumors; and polypeptides differentially expressed in certain tumors have utility as diagnostic tools.

The PTO's cited references are not contrary to Applicants' asserted utility

The PTO discusses Haynes *et al.* (Electrophoresis 1998; 19(11):1862-71) and Gygi *et al.* (Mol. and Cell. Bio., Mar. 1999; 1720-1730) in support for its argument that mRNA levels are not predictive of protein levels. Applicants have discussed at length in previous responses why the Haynes and Gygi references are not relevant to the issue of whether changes in mRNA level for a particular gene leads to changes in protein level. Applicants will not repeat their arguments here.

However, in an attempt to illustrate why references which relate to static global levels of mRNA and protein across different genes are not relevant to this issue, Applicants offer the following illustration and analogy with the understanding that like all illustrations and analogies, they are not perfect and therefore do not represent any admissions or binding statements regarding Applicants' disclosure or invention.

Haynes and Gygi discuss whether there is a correlation between the static level of mRNAs and proteins globally, *i.e.* across different genes. For example, in Experiment 1, if a particular cell type has 100 copies of mRNA for gene X, 200 copies of mRNA for gene Y, and 400 copies of mRNA for gene Z, the ratio of the amount of proteins X:Y:Z would be 1:2:4, such that there is a correlation between static levels of mRNA and protein across genes. This is essentially what the cited references examined. In contrast, Applicants are relying on a correlation between changes in mRNA level for a particular gene leading to a corresponding change in the level of the encoded protein. For example, in Experiment 2, if gene X has 100 copies of mRNA per cell in condition A (*e.g.* normal), and 200 copies of mRNA for gene X in condition B (*e.g.* tumor), the ratio of the amount of protein X in condition A:B would be 1:2, such that there is a correlation between the change in the level of mRNA and protein for a particular gene.

The PTO would like to argue that because there is no correlation between static levels of mRNA and protein across genes, as illustrated by Experiment 1, one of skill in the art would not expect an increase or decrease in the amount of mRNA for a particular gene to result in a

corresponding change in the amount of the encoded protein, as illustrated in Experiment 2. This is simply wrong.

Applicants emphasize, and the PTO will recognize, that this is just a simplified illustration to demonstrate the difference between the two issues being examined. However, this illustration makes clear that even if there is no correlation in the first experiment looking at static levels across genes, there can still be a correlation between changes in mRNA and protein for a particular gene as examined in the second experiment.

The Examiner also discusses the Chen, Alberts, Lewin, Zhigang, Meric, Jang, Hanash S [a] and Hanash et al. [b] references, stating that these references underscore the unpredictability in the art and disclose that the predictability of protein translation and its possible use as a diagnostic are not necessarily contingent on the levels of mRNA expression due to the multitude of homeostatic factors affecting transcription and translation. Applicants have addressed these references at length in previous responses, and will not repeat their arguments here.

Taken as a whole, Applicants maintain that the references discussed by the PTO do not support the PTO's rejection of Applicants' assertion that more often than not, there is a correlation between changes in mRNA level and changes in the level of the corresponding protein. If anything, the cited references support Applicants' position.

Applicants' previously submitted supporting declarations and references

In support of the assertion that changes in mRNA are positively correlated to changes in protein levels, Applicants previously submitted a copy of a second Declaration by J. Christopher Grimaldi, a copy of the declaration of Paul Polakis, Ph.D., excerpts from Molecular Biology of the Cell, a leading textbook in the field (Bruce Alberts, *et al.*, Molecular Biology of the Cell (3rd ed. 1994), and (4th ed. 2002), excerpts from the textbook, Genes VI, (Benjamin Lewin, Genes VI (1997)), a reference by Zhigang *et al.*, World Journal of Surgical Oncology 2:13, 2004, and a reference by Meric *et al.*, Molecular Cancer Therapeutics, vol. 1, 971-979 (2002). The details of these teachings, and how they support Applicants' asserted utility, are of record and will not be repeated here.

Applicants' additional supporting references

In addition to the supporting references previously submitted by Applicants, Applicants submit the following references to further support the assertion that changes in mRNA levels generally lead to corresponding changes in the level of the encoded polypeptide.

In a comprehensive study by Orntoft *et al.* (Mol. Cell. Proteomics. 2002; 1(1):37-45) (previously submitted with IDS, attached hereto as Exhibit 2), the authors examined gene amplification, mRNA expression level, and protein expression in pairs of non-invasive and invasive human bladder tumors. *Id.* at Abstract. The authors examined 40 well resolved abundant known proteins, and found that "[i]n general there was a highly significant correlation ($p<0.005$) between mRNA and protein alterations. Only one gene showed disagreement between transcript alteration and protein alteration." *Id.* at 42, col. 2. The alternations in mRNA and protein included both increases and decreases. *Id.* at 43, Table II. Clearly, a correlation in 39 of 40 genes examined supports Applicants' assertion that changes in mRNA level generally lead to corresponding changes in protein level.

In a study by Wang *et al.* (Urol. Res. 2000; 28(5):308-15) (abstract attached as Exhibit 3) the authors report that down-regulation of E-cadherin protein has been shown in various human tumors. *Id.* at Abstract. In the reported study, the authors examined the expression of cadherins and associated catenins at the mRNA level in paired tumor and nonneoplastic primary prostate cultures. They report that "[s]ix of seven cases of neoplastic cultures showed moderately-to-markedly decreased levels of E-cadherin and P-cadherin mRNA. Similar losses of alpha-catenin and beta-catenin mRNA were also observed." *Id.* As Applicants' assertion would predict, the authors state that the mRNA measures showed "good correlation" with the results from protein measures. The authors conclude by stating that "this paper presents a coordinated down-regulation in the expression of E-cadherin and associated catenins at the mRNA and protein level in most of the cases studied." *Id.*

In a more recent study by Munaut *et al.* (Int. J. Cancer. 2003; 106(6):848-55) (abstract attached as Exhibit 4) the authors report that vascular endothelial growth factor (VEGF) is expressed in 64-95% of glioblastomas (GBMs), and that VEGF receptors (VEGFR-1, its soluble form sVEGFR-1, VEGFR-2 and neuropilin-1) are expressed predominantly by endothelial cells. *Id.* at Abstract. The authors explain that infiltrating tumor cells and newly-formed capillaries

progress through the extracellular matrix by local proteolysis involving matrix metalloproteinases (MMPs). In the present study, the authors "used quantitative RT-PCR, Western blot, gelatin zymography and immunohistochemistry to study the expression of VEGF, VEGFR-1, VEGFR-2, sVEGFR-1, neuropilin-1, MT1-MMP, MMP-2, MMP-9 and TIMP-2 in 20 human GBMs and 5 normal brains. The expression of these MMPs was markedly increased in most GBMs with excellent correlation between mRNA and protein levels." *Id.* Thus, the results support Applicants' assertion that changes in mRNA level lead to corresponding changes in protein level.

In another recent study, Hui *et al.* (Leuk. Lymphoma. 2003; 44(8):1385-94 (abstract attached as Exhibit 5) used real-time quantitative PCR and immunohistochemistry to evaluate cyclin D1 mRNA and protein expression levels in mantle cell lymphoma (MCL). *Id.* at Abstract. The authors report that seven of nine cases of possible MCL showed overexpression of cyclin D1 mRNA, while two cases showed no cyclin D1 mRNA increase. *Id.* Similarly, "[s]ix of the seven cyclin D1 mRNA overexpressing cases showed increased cyclin D1 protein on tissue array immunohistochemistry; one was technically suboptimal." *Id.* The authors conclude that the study "demonstrates good correlation and comparability between measure of cyclin D1 mRNA ... and cyclin D1 protein." *Id.* Thus, this reference supports Applicants' assertion.

In a recent study by Khal *et al.* (Int. J. Biochem. Cell Biol. 2005; 37(10):2196-206) (abstract attached as Exhibit 6) the authors report that atrophy of skeletal muscle is common in patients with cancer and results in increased morbidity and mortality. *Id.* at Abstract. To further understand the underlying mechanism, the authors studied the expression of the ubiquitin-proteasome pathway in cancer patient muscle using a competitive RT-PCR to measure expression of mRNA for proteasome subunits C2 and C5, while protein expression was determined by western blotting. "Overall, both C2 and C5 gene expression was increased by about three-fold in skeletal muscle of cachectic cancer patients (average weight loss 14.5+/-2.5%), compared with that in patients without weight loss, with or without cancer. ... There was a good correlation between expression of proteasome 20Salpha subunits, detected by western blotting, and C2 and C5 mRNA, showing that increased gene expression resulted in increased protein synthesis." These findings support Applicants' assertion that changes in mRNA level lead to changes in protein level.

Maruyama *et al.* (Am. J. Patho. 1999; 155(3):815-22) (abstract attached as Exhibit 7) investigated the expression of three Id proteins (Id-1, Id-2 and Id-3) in normal pancreas, in pancreatic cancer and in chronic pancreatitis (CP). The authors report that pancreatic cancer cell lines frequently coexpressed all three Ids, "exhibiting good correlation between Id mRNA and protein levels." *Id.* at Abstract. In addition, the authors teach that all three Id mRNA levels were expressed at high levels in pancreatic cancer samples compared to normal or CP samples. At the protein level, Id-1 and Id-2 staining was faint in normal tissue, while Id-3 ranged from weak to strong. In contrast, in the cancer tissues "many of the cancer cells exhibited abundant Id-1, Id-2, and Id-3 immunoreactivity," and Id-1 and Id-2 protein was increased significantly in the cancer cells by comparison to the respective controls, mirroring the overexpression at the mRNA level. Thus, the authors report that in both cell lines and tissue samples, increased mRNA levels leads to an increase in protein overexpression, supporting Applicants' assertion.

Support for Applicants' assertion is also found in an article by Caberlotto *et al.* (Neurosci. Lett. 1999; 256(3):191-4) (abstract attached as Exhibit 8). In a previous study, the authors investigated alterations of neuropeptide Y (NPY) mRNA expression in the Flinders Sensitive Line rats (FSL), an animal model of depression. *Id.* at Abstract. The authors reported that in the current study, that NPY-like immunoreactivity (NPY-LI) was decreased in the hippocampal CA region, and increased in the arcuate nucleus, and that fluoxetine treatment elevated NPY-LI in the arcuate and anterior cingulate cortex. The authors state that "[t]he results demonstrate a good correlation between NPY peptide and mRNA expression." Thus, increases and decreases in mRNA levels were reflected in corresponding changes in protein level.

Misrachi and Shemesh (Biol. Reprod. 1999; 61(3):776-84) (abstract attached as Exhibit 9) investigated their hypothesis that FSH regulates the bovine cervical prostaglandin E(2) (PGE(2)) synthesis that is known to be associated with cervical relaxation and opening at the time of estrus. *Id.* at Abstract. Cervical tissue from pre-estrous/estrous, luteal, and postovulatory cows were examined for the presence of bovine (b) FSH receptor (R) and its corresponding mRNA. The authors report that bFSHR mRNA in the cervix was maximal during pre-estrus/estrus, and that the level of FSHR protein was significantly higher in pre-estrous/estrous cervix than in other cervical tissues. *Id.* The authors state that "[t]here was a good correlation between the 75-kDa protein expression and its corresponding transcript of 2.55

kb throughout the estrous cycle as described by Northern blot analysis as well as RT-PCR." *Id.* Thus, changes in the level of mRNA for bFSHR led to corresponding changes in FSHR protein levels, a result which supports Applicants' assertion.

In a study by Stein *et al.* (J. Urol. 2000; 164(3 Pt 2):1026-30) (abstract attached as Exhibit 10), the authors studied the role of the regulation of calcium ion homeostasis in smooth muscle contractility. *Id.* at Abstract. The authors investigated the correlation between sarcoplasmic endoplasmic reticulum, calcium, magnesium, adenosine triphosphatase (SERCA) protein and gene expression, and the contractile properties in the same bladder. Partial bladder outlet obstructions were created in adult New Zealand white rabbits, which were divided into control, sham operated and obstructed groups. Stein *et al.* report that "[t]he relative intensities of signals for the Western [protein] and Northern [mRNA] blots demonstrated a strong correlation between protein and gene expression. ... The loss of SERCA protein expression is mediated by down-regulation in gene expression in the same bladder." *Id.* This report supports Applicants' assertion that changes in mRNA level, e.g. a decrease, lead to a corresponding change in the level of the encoded protein, e.g. a decrease.

In an article by Gou and Xie (Zhonghua Jie He He Hu Xi Za Zhi. 2002; 25(6):337-40) (abstract attached as Exhibit 11) the authors investigated the expression of macrophage migration inhibitory factor (MIF) in human acute respiratory distress syndrome(ARDS) by examining the expression of MIF mRNA and protein in lung tissue in ARDS and normal persons. *Id.* at Abstract. The authors report "undetectable or weak MIF mRNA and protein expression in normal lungs. In contrast, there was marked upregulation of MIF mRNA and protein expression in the ARDS lungs." *Id.* This is consistent with Applicants' assertion that a change in mRNA for a particular gene, e.g. an increase, generally leads to a corresponding change in the level of protein expression, e.g. an increase.

These studies are representative of numerous published studies which support Applicants' assertion that changes in mRNA level generally lead to corresponding changes in the level of the expressed protein. Applicants submit herewith an addition 70 references (abstracts attached as Exhibit 12) which support Applicants' assertion.

In addition to these supporting references, Applicants also submit herewith additional references which offer indirect support of Applicants' asserted utility. As discussed above,

Applicants have challenged the relevance of references such as Haynes *et al.* and Gygi *et al.*, which do not attempt to examine the correlation between a change in mRNA level and a change in the level of the corresponding protein level. Because the PTO continues to rely on these references, Applicants are submitting references which report results that are contrary to the PTO's cited references and offer indirect support for Applicants' asserted utility.

For example, in an article by Futcher *et al.* (Mol. Cell Biol. 1999; 19(11):7357-68) (abstract attached as Exhibit 13) the authors conducted a study of mRNA and protein expression in yeast which was nearly identical to the one conducted by Gygi *et al.* Contrary to the results of the earlier study by Gygi, Futcher *et al.* report "a good correlation between protein abundance, mRNA abundance, and codon bias." *Id.* at Abstract.

In a study which is more closely related to Applicants' asserted utility, Godbout *et al.* (J. Biol. Chem. 1998; 273(33)21161-8) (abstract attached as Exhibit 14) studied the DEAD box gene, DDX1, in retinoblastoma and neuroblastoma tumor cell lines. The authors report that "there is a good correlation with DDX1 gene copy number, DDX1 transcript levels, and DDX1 protein levels in all cell lines studied." *Id.* Thus, in these cancer cell lines, DDX1 mRNA and protein levels are correlated.

Similarly, in an article by Papotti *et al.* (Virchows Arch. 2002; 440(5):461-75) (abstract attached as Exhibit 15) the authors examined the expression of three somatostatin receptors (SSTR) at the mRNA and protein level in forty-six tumors. *Id.* at Abstract. The authors report a "good correlation between RT-PCR [mRNA level] and IHC [protein level] data on SSTR types 2, 3, and 5." *Id.*

Van der Wilt *et al.* (Eur. J. Cancer. 2003; 39(5):691-7) (abstract attached as Exhibit 16) studied deoxycytidine kinase (dCK) in seven cell lines, sixteen acute myeloid leukemia samples, ten human liver samples, and eleven human liver metastases of colorectal cancer origin. *Id.* at Abstract. The authors report that "enzyme activity and protein expression levels of dCK in cell lines were closely related to the mRNA expression levels" and that there was a "good correlation between the different dCK measurements in malignant cells and tumors." *Id.*

Grenback *et al.* (Regul. Pept. 2004; 117(2):127-39) (abstract attached as Exhibit 17) studied the level of galanin in human pituitary adenomas using a specific radioimmunoassay. *Id.*

at Abstract. The authors report that "[i]n the tumors analyzed with in situ hybridization there was a good correlation between galanin peptide levels and galanin mRNA expression." *Id.*

Similarly, Shen *et al.* (Blood. 2004; 104(9):2936-9) (abstract attached as Exhibit 18) examined the level of B-cell lymphoma 2 (BCL2) protein expression in germinal center (GC) B-cells and diffuse large B-cell lymphoma (DLBCL). *Id.* at Abstract. The authors report that "GC cells had low expression commensurate with the low protein expression level" and that in DLBCL the level of BCL2 mRNA and protein expression showed "in general, a good correlation." *Id.*

Likewise, in an article by Fu *et al.* (Blood 2005; 106(13):4315-21) (abstract attached as Exhibit 19) the authors report that six mantle cell lymphomas studied "expressed either cyclin D2 (2 cases) or cyclin D3 (4 cases)." *Id.* at Abstract. "There was a good correlation between cyclin D protein expression and the corresponding mRNA expression levels by gene expression analysis." *Id.*

These examples are only a few of the many references Applicants could cite in rebuttal to the PTO's arguments. Applicants submit herewith 26 additional references (abstracts attached as Exhibit 20) which also support Applicants' assertion in that they report a correlation between the level of mRNA and corresponding protein, contrary to the assertion of the PTO that mRNA and protein levels are not correlated.

In summary, Applicants submit herewith a total of 113 references in addition to the declarations and references already of record which support Applicants' asserted utility, either directly or indirectly. These references support the assertion that in general, a change in mRNA expression level for a particular gene leads to a corresponding change in the level of expression of the encoded protein. As Applicants have previously acknowledged, the correlation between changes in mRNA level and protein level is not exact, and there are exceptions (*see, e.g.*, abstracts attached as Exhibit 21). However, Applicants remind the PTO that the asserted utility does not have to be established to a statistical certainty, or beyond a reasonable doubt. *See M.P.E.P.* at § 2107.02, part VII (2004). Therefore, the fact that there are exceptions to the correlation between changes in mRNA and changes in protein does not provide a proper basis for rejecting Applicants' asserted utility. Applicants submit that considering the evidence as a whole, with the overwhelming majority of the evidence supporting Applicants' asserted utility, a

person of skill in the art would conclude that Applicants' asserted utility is "more likely than not true." *Id.*

Applicants also previously submitted the Polakis Declaration in support of their position that, in general, changes in mRNA levels correlate with changes in protein levels. The Eaxmienr has stated that the Polakis Declaration is not persuasive, because, among other things, "Dr. Polakis refers to facts; however, the data is not included in the declaration so the examiner could not independently evaluate them." *Final Office Action* at 18. Without acquiescing to the propriety of this rejection, and merely to expedite prosecution in this case, Applicants submit herewith as Exhibit 22 a second Declaration by Dr. Polakis (Polakis II) that presents evidentiary data in Exhibit B. Exhibit B of the Declaration identifies 28 gene transcripts out of 31 gene transcripts (i.e., greater than 90%) that showed good correlation between tumor mRNA and tumor protein levels. As Dr. Polakis' Declaration (Polakis II) says "[a]s such, in the cases where we have been able to quantitatively measure both (i) mRNA and (ii) protein levels in both (i) tumor tissue and (ii) normal tissue, we have observed that in the vast majority of cases, there is a very strong correlation between increases in mRNA expression and increases in the level of protein encoded by that mRNA." Accordingly, Dr. Polakis has provided the facts to enable the Examiner to draw independent conclusions.

The case law has clearly established that in considering affidavit evidence, the PTO must consider all of the evidence of record anew. *In re Rinehart,* 531 F.2d 1084, 189 USPQ 143 (C.C.P.A. 1976) and *In re Piasecki,* 745 F.2d. 1015, 226 USPQ 881 (Fed. Cir. 1985). "After evidence or argument is submitted by the applicant in response, patentability is determined on the totality of the record, by a preponderance of the evidence with due consideration to persuasiveness of argument." *In re Alton,* 37 U.S.P.Q.2d 1578, 1584 (Fed. Cir. 1996)(quoting *In re Oetiker,* 977 F.2d 1443, 1445, 24 U.S.P.Q.2d 1443, 1444 (Fed. Cir. 1992)). Furthermore, the Federal Court of Appeals held in *In re Alton*, "We are aware of no reason why opinion evidence relating to a fact issue should not be considered by an examiner." *Id.* at 1583. Applicants also respectfully draw the PTO's attention to the Utility Examination Guidelines which state, "Office personnel must accept an opinion from a qualified expert that is based upon relevant facts whose accuracy is not being questioned; it is improper to disregard the opinion solely because of a

disagreement over the significance or meaning of the facts offered." Part IIB, 66 Fed. Reg. 1098 (2001).

The PTO has asserted that the state of the art is such that polypeptide levels cannot be accurately predicted from mRNA levels. Applicants have addressed each of the PTO's supporting references and shown that they are either irrelevant, or taken as a whole, actually support Applicants' assertion that a change in mRNA level leads to a corresponding change in the level of the encoded protein. In addition, Applicants have submitted expert declarations, textbook excerpts, and over 115 scientific publications which support Applicants' arguments.

In conclusion, Applicants submit that they have offered sufficient evidence to establish that it is more likely than not that one of skill in the art would believe that because the PRO180 mRNA is differentially expressed in rectal and lung tumor tissue, the PRO180 polypeptide will likewise be differentially expressed in these tumors. This differential expression of the PRO180 polypeptide makes the claimed polypeptides useful as diagnostic tools for cancer, particularly rectal and lung tumor.

The Arguments made by the PTO are not Sufficient to satisfy the PTO's Initial Burden of Offering Evidence "that one of ordinary skill in the art would reasonably doubt the asserted utility"

As stated above, an Applicant's assertion of utility creates a presumption of utility that will be sufficient to satisfy the utility requirement of 35 U.S.C. § 101, "unless there is a reason for one skilled in the art to question the objective truth of the statement of utility or its scope." *In re Langer*, 503 F.2d 1380, 1391, 183 USPQ 288, 297 (CCPA 1974). The evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is a preponderance of the evidence, or "more likely than not" standard. *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). This is stated explicitly in the M.P.E.P.:

> [T]he applicant does not have to provide evidence sufficient to establish that an asserted utility is true "beyond a reasonable doubt." **Nor must the applicant provide evidence such that it establishes an asserted utility as a matter of statistical certainty**. Instead, evidence will be sufficient if, considered as a whole, it leads a person of ordinary skill in the art to conclude that the asserted utility is more likely than not true. M.P.E.P. at § 2107.02, part VII (2004) (underline emphasis in original, bold emphasis added, internal citations omitted).

The PTO has the initial burden to offer evidence "that one of ordinary skill in the art would reasonably doubt the asserted utility." *In re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995). Only then does the burden shift to the Applicant to provide rebuttal evidence. *Id.* As stated in the M.P.E.P., such rebuttal evidence does not need to absolutely prove that the asserted utility is real. Rather, the evidence only needs to be reasonably indicative of the asserted utility.

Applicants remind the PTO that the M.P.E.P. cautions that rejections for lack of utility are rarely sustained by federal courts, and that generally speaking, a utility rejection was sustained because the applicant asserted a utility "that could only be true if it violated a scientific principle, such as the second law of thermodynamics, or a law of nature, or was wholly inconsistent with contemporary knowledge in the art." M.P.E.P. § 2107.02 III B., citing *In re Gazave*, 379 F.2d 973, 978, 154 U.S.P.Q. 92, 96 (CCPA 1967) (emphasis in original). Rather than being wholly inconsistent with contemporary knowledge in the art, Applicants' asserted utility is squarely within the teaching of leading textbooks in the field, and is supported by numerous references and the declarations of skilled experts.

Applicants' asserted utility is based on the assertion that changes in mRNA level generally result in corresponding changes in the level of the encoded protein. In rejecting this conclusion, the PTO has cited references by Hu *et al.*, Gygi *et al.*, and others.

As explained above, these references are largely irrelevant when determining whether Applicants' asserted utility is more likely than not true. Given the lack of support for the PTO's position, Applicants submit that the PTO has not met its initial burden of overcoming the presumption that the asserted utility is sufficient to satisfy the utility requirement. And even if the PTO has met that burden, the Applicants' supporting rebuttal evidence, including two uncontested expert declarations, excerpts from three textbooks, and over 115 scientific articles, is more than sufficient to establish that one of skill in the art would be more likely than not to believe that the claimed polypeptides can be used as diagnostic tools for cancer, particularly rectal and lung cancer.

Conclusion

The PTO has asserted that the state of the art is such that polypeptide levels cannot be accurately predicted from mRNA levels. Applicants have addressed each of the PTO's supporting references and shown that they are either irrelevant, or taken as a whole, actually support Applicants' assertion that a change in mRNA level leads to a corresponding change in the level of the encoded protein. In addition, Applicants have submitted expert declarations, textbook excerpts, and over 115 scientific publications which support Applicants' asserted utility.

Given the totality of the evidence provided, Applicants submit that they have established a substantial, specific, and credible utility for the claimed polypeptides as diagnostic tools. According to the PTO Utility Examination Guidelines (2001), irrefutable proof of a claimed utility is not required. Rather, a specific, substantial, and credible utility requires only a "reasonable" confirmation of a real world context of use. Applicants remind the PTO that:

> A small degree of utility is sufficient . . . The claimed invention must only be capable of performing some beneficial function . . . An invention does not lack utility merely because the particular embodiment disclosed in the patent lacks perfection or performs crudely... A commercially successful product is not required... Nor is it essential that the invention accomplish all its intended functions... or operate under all conditions... partial success being sufficient to demonstrate patentable utility... In short, the defense of non-utility cannot be sustained without proof of total incapacity. If an invention is only partially successful in achieving a useful result, a rejection of the claimed invention as a whole based on a lack of utility is not appropriate. M.P.E.P. at 2107.01 (emphasis in original, citations omitted).

Applicants submit that they have established that it is more likely than not that one of skill in the art would reasonably accept the utility for the claimed PRO180 polypeptides set forth in the specification. In view of the above, Applicants respectfully request that the PTO reconsider and withdraw the utility rejection under 35 U.S.C. §101.

Rejections under 35 U.S.C. § 112, first paragraph – Enablement

The PTO maintains its rejection of Claims 44-45, 47 and 49-55 as lacking enablement. The PTO states that because the claimed invention is not supported by either a substantial

asserted utility or a well established utility, one skilled in the art would not know how to use the claimed invention.

Applicants submit that in the discussion of the 35 U.S.C. § 101 rejection above, Applicants have established a substantial, specific, and credible utility for the claimed polypeptides. Applicants respectfully request that to the extent the enablement rejection is based on a lack of utility, the PTO reconsider and withdraw the enablement rejection under 35 U.S.C. §112, first paragraph.

The PTO also maintains the rejection of Claims 42-43 and 50-55 because the claims allegedly contain subject matter, which was not described in the specification in such a way as to enable one skilled in the art to make and/or use the invention. The PTO maintains that "applicant has not provided sufficient guidance and direction to assist to enable one of ordinary skill in the art to make and use the claimed protein variants that are 95% or 99% identical to SEQ ID NO:2...." *Final Office Action* at 26.

As an initial matter, Applicants note that 42 and 43 have been cancelled, and pending Claim 50 has been amended to depend from Claim 44. Claim 51 ultimately depends from Claim 44 as well. Any arguments based on a failure to enable variants are not applicable to pending Claims 50 and 51.

As for Claims 52-55, the specification teaches in detail how to make the claimed polypeptides, including variants thereof, and antibodies which specifically bind PRO180. Likewise, the specification provides sufficient guidance as to how to use the claimed polypeptides. Thus, contrary to the PTO's statement, there is significant guidance how to make and use the claimed polypeptides. In addition, as the disclosure and references cited in the specification make clear, the production of polypeptides, polypeptide variants, and specific antibodies is a predictable and well established aspect of the biological sciences. *See, e.g., In re Wands*, 858 F.2d 731, 8 U.S.P.Q. 2d 1400 (Fed. Cir. 1988) (reversing the Board's decision of non-enablement and holding that as of 1980, undue experimentation was not required to make high-affinity monoclonal antibodies to a target peptide).

In conclusion, the PTO's rejection based on lack of utility has been addressed above, and the PTO has otherwise failed to meet its burden to establish a reasonable basis to question the enablement provided for the claimed invention. Given the skill in the art and the disclosure of

how to make and use the claimed polypeptides, Applicants request that the PTO reconsider and withdraw its rejections under 35 U.S.C. § 112, first paragraph.

Rejection under 35 U.S.C. § 112, first paragraph – Written Description

The PTO rejects Claims 42-43 and 52-55 under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. The PTO asserts that the claims contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors, at the time the application was filed, had possession of the claimed invention.

The PTO has Failed to Meet Its Initial Burden of Rebutting the Presumption that the Pending Claims are Adequately Described

To overcome the presumption that the claimed subject matter is adequately described, the PTO must present "evidence why a person skilled in the art would not recognize in an applicant's disclosure a description of the invention defined by the claims. *Wertheim*, 541 F.2d at 263, 191 U.S.P.Q. at 97." *M.P.E.P.* § 2163.04. The PTO has asserted that "the specification does not disclose any polypeptides that are 95% or 99% identical to SEQ ID NO:2 and is more highly expressed in rectal tumors or normal lung compared to normal rectum or lung tumors. Also, unlike Example 14 of the written description training materials, the polypeptide of SEQ ID NO:2 is not disclosed as having any particular function or biological activity." *Final Office Action* at 23.

The Pending Claims are Adequately Described

As mentioned above, pending Claims 42-43 have been cancelled. As for pending Claims 52-55, Applicants maintain that these claims are analogous to the claims discussed in Example 14 of the written description training materials available on the PTO's website. In Example 14, the written description requirement was found to be satisfied for claims directed to polypeptides with 95% homology to a disclosed sequence that also possess a recited catalytic activity, where procedures for making variant proteins were routine in the art and the specification provided an assay for detecting the recited catalytic activity of the protein. This disclosure satisfies the written description requirement even though the applicant had disclosed only a single species and

had not made any variants. The Guidelines state that "[t]he single species disclosed is representative of the genus because all members have at least 95% structural identity with the reference compound and because of the presence of an assay which applicant provided for identifying all of the at least 95% identical variants of SEQ ID NO: 3 which are capable of the specified catalytic activity."

Like Example 14, Claims 52-55 have very high sequence homology to the disclosed sequence and must share an epitope sufficient to generate antibodies which specifically detect the polypeptide of SEQ ID NO:2 in rectum and lung tissue samples. As in Example 14, at the time of the effective filing date of the instant application, it was well known in the art how to make polypeptides with at least 95% amino acid sequence identity to the disclosed sequences. *See, e.g., Specification* at ¶¶ [0256]-[0271]. In addition, the specification discloses in detail how to make antibodies which specifically detect a particular PRO polypeptide, and how to use them to detect the PRO polypeptide in a particular tissue. *See, e.g., Specification* ¶¶ [0363]-[0379], [0407], and [0493]-[0499]. Like a particular catalytic activity, the function of being useful to produce an antibody specific to SEQ ID NO:2 is directly related to the structure of the claimed polypeptides. Thus, like Example 14, the genus of polypeptides that have at least 95% amino acid sequence identity to the disclosed sequences and possess the described functional activity are adequately described.

Applicant's maintain that the Federal Circuit decision in *In re Wallach*, 378 F.3d 1330, 1333-34 (Fed. Cir. 2004) is applicable here. The Court did not require the applicants in *Wallach* to actually make or individually describe all of the vast number of sequences which encode the disclosed sequence. This is in spite of the fact that only a single sequence was disclosed, and the encompassed genus was enormous due to codon degeneracy in the genetic code – even the most skilled artisan could not individually envision the detailed chemical structure of the nucleic acids encompassed by the claimed genus. The Court reasoned that because it is routine to convert between amino acid sequences to nucleic acid sequences, disclosure of a single amino acid sequence was sufficient to place the applicants in possession of the enormous genus of nucleic acids which could encode the sequence.

The facts in *Wallach* are very similar to the instant case. Here, Applicants have disclosed SEQ ID NO:2, and claim polypeptides which are at least 95% identical to it and have the

functional limitation of the ability to generate antibodies which can be used to specifically detect SEQ ID NO:2 in rectum and lung tissue samples. As discussed above, it is routine in the art to create polypeptides which have at least 95% sequence identity to SEQ ID NO:2 – it is just as predictable and easy as creating all of the nucleic acids which encode a particular amino acid sequence. Similarly, it is well within the knowledge of those skilled in the art how to determine which polypeptides can be used to make the recited antibodies. The predictability of this structure/function combination is sufficient to place the claimed subject matter in the possession of the Applicants, and thus the claimed polypeptides are adequately described. The *Wallach* opinion makes clear that there is no need to literally describe more than a single species to adequately describe a large genus where one of skill in the art recognizes that the disclosed species puts the applicant in possession of the claimed genus.

Further, the PTO has not provided any reasoning or evidence as to how the absence of the disclosure of "biological activity" results in an inadequate description of the subject matter of Claims 52-55. The claimed subject matter relates to polypeptides that have at least 95% sequence identity to SEQ ID NO:2, and can be used to generate an antibody which can be used to specifically detect the polypeptide of SEQ ID NO:2 in lung or rectum tissue samples. Applicants fail to see how any "biological activity" of the claimed polypeptides, aside from being able to generate an antibody which can specifically detect the polypeptide of SEQ ID NO:2, is at all relevant to an adequate description of the claimed polypeptides which are not claimed on the basis of any "biological activity." In the absence of any other arguments as to why one of skill in the art would not recognize a description of the claimed invention in Applicants' disclosure, the PTO has failed to rebut the presumption that the specification satisfies the written description requirement for Claims 52-55. *See M.P.E.P.* § 2163.04.

In conclusion, Applicants submit that they have satisfied the written description requirement for the pending claims based on the actual reduction to practice of SEQ ID NO:2, by specifying a high level of amino acid sequence identity, and by describing how to make antibodies to the disclosed sequence, all of which result in a lack of substantial variability in the species falling within the scope of the instant claims. Applicants submit that this disclosure would allow one of skill in the art to "recognize that the applicant was in possession of the necessary common attributes or features of the elements possessed by the members of the genus."

Hence, Applicants respectfully request that the PTO reconsider and withdraw the written description rejection under 35 U.S.C. §112.

Rejection under 35 U.S.C. § 112, first paragraph – New Matter

The PTO maintains its rejection of Claims 42-43 and 50-55 as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors, at the time the application was filed, had possession of the claimed invention.

Claims 42-43 have been cancelled, and Claims 50-51 now depend from Claim 44. Thus, Applicants assert that this rejection is inapplicable to these claims. As for Claims 52-55, the PTO argues that "the specification does not disclose or contemplate any polypeptide that is 95% or 99% identical to the polypeptide of SEQ ID NO:2 or amino acids 34-53, 114-121 or 181-266 of SEQ ID NO:2 and is more highly expressed in normal kidney or rectal tumor compared to lung tumor or normal rectum....Thus, there is insufficient written support for the claimed genus of variant PRO180 polypeptides that are more highly expressed in normal kidney or rectal tumor compared to lung tumor or normal rectum as encompassed by the claims." *Final Office Action* at 27-28. Applicants point out that none of Claims 52-55 recite "variant PRO180 polypeptides that are more highly expressed in normal kidney or rectal tumor compared to lung tumor or normal rectum." Rather, these claims are directed to polypeptides that are at least 95% identical to SEQ ID NO:2 and have the functional limitation of the ability to generate antibodies which can be used to specifically detect SEQ ID NO:2 in rectum and lung tissue samples. As noted above, Applicants submit that they have satisfied the written description requirement for the pending claims based on the actual reduction to practice of SEQ ID NO:2, by specifying a high level of amino acid sequence identity, by describing how to make antibodies to the disclosed sequence, and by describing how to test tissue samples for expression of a PRO polypeptide. Accordingly, Applicants request that the rejection of Claims 50-55 be withdrawn.

Priority

Applicants acknowledge that the PTO has granted the present application priority to PCT/US00/23328, filed 24 August 2000, but maintain that for the reasons of record, they are also entitled to priority to U.S. Provisional Application No. 60/096,012 filed on August 10, 1998.

Rejections under 35 U.S.C. § 102 – Anticipation

Feng et al.

Claims 44-45, 47 and 49-55 are rejected under 35 U.S.C. 102(b) on the assertion that they are anticipated by Feng et al (WO 99/24836, published May 1999).

Applicants maintain that they are entitled to priority to U.S. Provisional Application No. 60/096,012 filed on **August 10, 1998**. This application includes the disclosure of the full length sequence of SEQ ID NOs:1 and 2. Applicants demonstrated, by means of the disclosure in their provisional application filed August 10, 1998, that they were in possession of so much of the claimed invention, i.e. SEQ ID NO:2, as disclosed in the Feng et al. reference dated May 1999. As stated by the PTO, "a chemical composition and its properties are inseparable. Therefore, if the prior art teaches the identical chemical structure, the properties applicant discloses and/or claims are necessarily present." *Office Action dated 09/06/2005* at 21. Applying this reasoning, Applicants provided the chemical composition of SEQ ID NO:2 as of August 10, 1998. They necessarily therefore also provided the properties of SEQ ID NO:2 as of August 10, 1998. The Feng et al. reference, published May 1999, cannot therefore anticipate the claimed invention.

In addition, Applicants maintain that the *In re Stempel* and *In re Moore* cases support their position that the cited Feng et al. reference is not available as prior art.

Finally, Applicants maintain that the Declaration under 37 CFR §1.131, which was previously submitted on December 9, 2004, establishes that the presently claimed invention antedates the Feng et al. reference. The Declaration and the supporting evidence submitted therewith established conception of the invention prior to the May 1999 publication date of Feng et al., and diligent reduction to practice thereafter. Withdrawal of the rejection under 35 U.S.C. § 102 is therefore respectfully requested.

Baker et al.

The PTO has rejected Claims 42-45, 47 and 49-55 as being anticipated by Baker et al. (WO 99/63088, published 12/99) under 35 U.S.C. § 102(a). The PTO asserts that the "the filing date for the purpose of art rejections is deemed to be 24 August 2000 because prior applications...do not disclose the quantitative PCR analysis of a cDNA library measuring mRNA

expression," and therefore rejects the Declaration of Goddard et al. filed under 37 C.F.R. 1.131. *Final Office Action* at 29-30.

35 U.S.C. § 102(a) states that a person is entitled to a patent unless "the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, **before the invention thereof by the applicant**..." The M.P.E.P. states that "A rejection based on 35 U.S.C. 102(a) can be overcome by:... **Filing an affidavit or declaration under 37 CFR 1.131 showing prior invention**...." *See* M.P.E.P. § 706.02 (b). As set forth in 37 C.F.R. § 1.131, a patent applicant "may submit an appropriate oath or declaration to establish invention of the subject matter of the rejected claim prior to the effective date of the reference or activity on which the rejection is based." *See also*, M.P.E.P. § 715. "The affidavit or declaration must state FACTS and produce such documentary evidence and exhibits in support thereof as are available to show conception and completion of the invention in this country ... **at least conception being at a date prior to the effective date of the reference**." *See* M.P.E.P. § 715.07. The showing of facts must be sufficient to show "**conception of the invention prior to the effective date of the reference coupled with due diligence from prior to the reference date to a subsequent (actual) reduction to practice.**" *See id.*

Applicants' 1.131 Declaration demonstrates that the claimed subject matter, more particularly the polypeptide of SEQ ID NO:2, was conceived by Applicants prior to May 1999. Furthermore, as evidenced by the Declaration and accompanying exhibits, Applicants exhibited diligence in reducing the subject matter of the claims to practice by performing various assays to confirm the function of the polypeptide.

Applicants have therefore provided a declaration showing prior invention of the claimed subject matter. The declaration establishes that the presently claimed subject matter was conceived prior to the December 1999 date of Baker et al, and diligently reduced to practice thereafter. Applicants have established prior invention by their 131 Declaration. This is sufficient to remove the cited reference as prior art.

New Ground for Rejection

Claim 47 is newly rejected as being indefinite for reciting "the extracellular domain." The PTO states that there is insufficient antecedent basis for this limitation.

Applicants have amended Claim 47, and request that the rejection be withdrawn.

CONCLUSION

In view of the above, Applicants respectfully maintain that claims are patentable and request that they be passed to issue. Applicants invite the Examiner to call the undersigned if any remaining issues may be resolved by telephone.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: June 30, 2006

By: _____

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EXHIBIT 1

Proteomics. 2005 Mar;5(4):894-906.

A transcriptomic and proteomic analysis of the effect of CpG-ODN on human THP-1 monocytic leukemia cells.

Kuo CC, Kuo CW, Liang CM, Liang SM.

Institute of BioAgricultural Sciences, Academia Sinica, Taipei, Taiwan.

The CpG motif of bacterial DNA (CpG-DNA) is a potent immunostimulating agent whose mechanism of action is not yet clear. Here, we used both DNA microarray and proteomic approaches to investigate the effects of oligodeoxynucleotides containing the CpG motif (CpG-ODN) on gene transcription and protein expression profiles of CpG-ODN responsive THP-1 cells. Microarray analysis revealed that 2 h stimulation with CpG-ODN up-regulated 50 genes and down-regulated five genes. These genes were identified as being associated with inflammation, antimicrobial defense, transcriptional regulation, signal transduction, tumor progression, cell differentiation, proteolysis and metabolism. Longer stimulation (8 h) with CpG-ODN enhanced transcriptional expression of 58 genes. Among these 58 genes, none except one, namely WNT1 inducible signaling pathway protein 2, was the same as those induced after 2 h stimulation. Proteomic analysis by two-dimensional gel electrophoresis, followed by mass spectrometry identified several proteins up-regulated by CpG-ODN. These proteins included heat shock proteins, modulators of inflammation, metabolic proteins and energy pathway proteins. Comparison of microarray and proteomic expression profiles showed poor correlation. Use of more reliable and sensitive analyses, such as reverse transcriptase polymerase chain reaction, Western blotting and functional assays, on several genes and proteins, nonetheless, confirmed that there is indeed good correlation between mRNA and protein expression after CpG-ODN treatment. This study also revealed that several anti-apoptotic and neuroprotective related proteins, not previously reported, are activated by CpG-DNA. These findings have extended our knowledge on the activation of cells by CpG-DNA and may contribute to further understanding of mechanisms that link innate immunity with acquired immune response(s).

PMID: 15693060 [PubMed - indexed for MEDLINE]

EXHIBIT 2

Genome-wide Study of Gene Copy Numbers, Transcripts, and Protein Levels in Pairs of Non-invasive and Invasive Human Transitional Cell Carcinomas*

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Gain and loss of chromosomal material is characteristic of bladder cancer, as well as malignant transformation in general. The consequences of these changes at both the transcription and translation levels is at present unknown partly because of technical limitations. Here we have attempted to address this question in pairs of non-invasive and invasive human bladder tumors using a combination of technology that included comparative genomic hybridization, high density oligonucleotide array-based monitoring of transcript levels (5600 genes), and high resolution two-dimensional gel electrophoresis. The results showed that there is a gene dosage effect that in some cases superimposes on other regulatory mechanisms. This effect depended ($p < 0.015$) on the magnitude of the comparative genomic hybridization change. In general (18 of 23 cases), chromosomal areas with more than 2-fold gain of DNA showed a corresponding increase in mRNA transcripts. Areas with loss of DNA, on the other hand, showed either reduced or unaltered transcript levels. Because most proteins resolved by two-dimensional gels are unknown it was only possible to compare mRNA and protein alterations in relatively few cases of well focused abundant proteins. With few exceptions we found a good correlation ($p < 0.005$) between transcript alterations and protein levels. The implications, as well as limitations, of the approach are discussed. *Molecular & Cellular Proteomics 1:37–45, 2002.*

Aneuploidy is a common feature of most human cancers (1), but little is known about the genome-wide effect of this phenomenon at both the transcription and translation levels. High throughput array studies of the breast cancer cell line BT474 has suggested that there is a correlation between DNA copy numbers and gene expression in highly amplified areas (2), and studies of individual genes in solid tumors have revealed a good correlation between gene dose and mRNA or protein levels in the case of c-erb-B2, *cyclin d1*, *ems1*, and N-myc (3–5). However, a high cyclin D1 protein expression has been observed without simultaneous amplification (4), and a low level of c-myc copy number increase was observed without concomitant c-myc protein overexpression (6).

In human bladder tumors, karyotyping, fluorescent *in situ* hybridization, and comparative genomic hybridization (CGH)[1] have revealed chromosomal aberrations that seem to be characteristic of certain stages of disease progression. In the case of non-invasive pTa transitional cell carcinomas (TCCs), this includes loss of chromosome 9 or parts of it, as well as loss of Y in males. In minimally invasive pT1 TCCs, the following alterations have been reported: 2q−, 11p−, 1q+, 11q13+, 17q+, and 20q+ (7–12). It has been suggested that these regions harbor tumor suppressor genes and oncogenes; however, the large chromosomal areas involved often contain many genes, making meaningful predictions of the functional consequences of losses and gains very difficult.

In this investigation we have combined genome-wide technology for detecting genomic gains and losses (CGH) with gene expression profiling techniques (microarrays and proteomics) to determine the effect of gene copy number on transcript and protein levels in pairs of non-invasive and invasive human bladder TCCs.

EXPERIMENTAL PROCEDURES

Material—Bladder tumor biopsies were sampled after informed consent was obtained and after removal of tissue for routine pathology examination. By light microscopy tumors 335 and 532 were staged by an experienced pathologist as pTa (superficial papillary).

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[1] The abbreviations used are: CGH, comparative genomic hybridization; TCC, transitional cell carcinoma; LOH, loss of heterozygosity; PA-FABP, psoriasis-associated fatty acid-binding protein; 2D, two-dimensional.

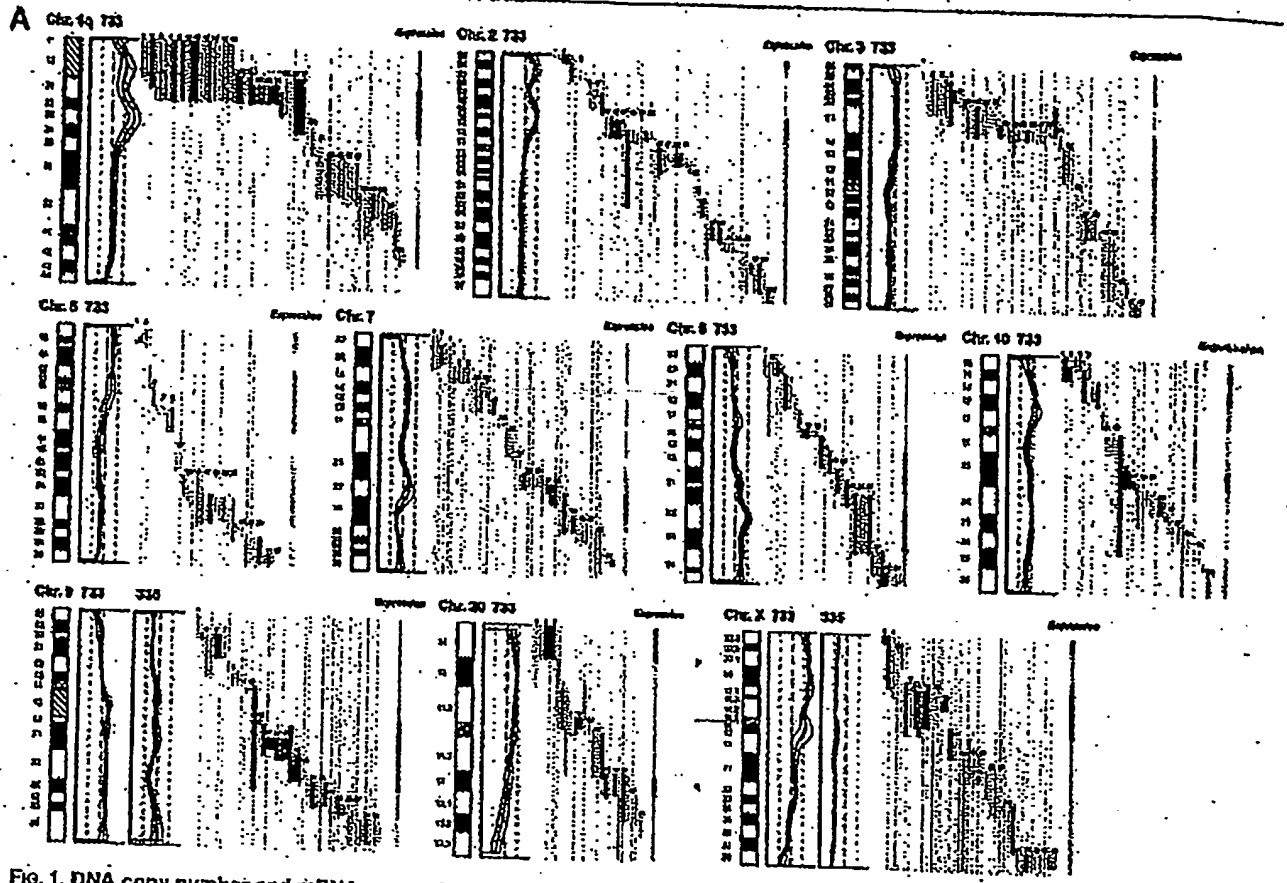

FIG. 1. DNA copy number and mRNA expression level. Shown from *left* to *right* are chromosome (*Chr.*), CGH profiles, gene location and expression level of specific genes, and overall expression level along the chromosome. *A*, expression of mRNA in invasive tumor 733 as compared with the non-invasive counterpart tumor 335. *B*, expression of mRNA in invasive tumor 827 compared with the non-invasive counterpart tumor 532. The average fluorescent signal ratio between tumor DNA and normal DNA is shown along the length of the chromosome (*left*). The *bold curve* in the ratio profile represents a mean of four chromosomes and is surrounded by *thin curves* indicating one standard deviation. The *central vertical line* (*broken*) indicates a ratio value of 1 (no change), and the *vertical lines* next to it (*dotted*) indicate a ratio of 0.5 (*left*) and 2.0 (*right*). In chromosomes where the non-invasive tumor 335 used for comparison showed alterations in DNA content, the ratio profile of that chromosome is shown to the *right* of the invasive tumor profile. The *colored bars* represents one gene each, identified by the running *numbers above* the *bars* (the name of the gene can be seen at www.MDL.DK/sdata.html). The *bars* indicate the purported location of the gene, and the *colors* indicate the expression level of the gene in the invasive tumor compared with the non-invasive counterpart; >2-fold increase (*black*), >2-fold decrease (*blue*), no significant change (*orange*). The *bar* to the *far right*, entitled *Expression* shows the resulting change in expression along the chromosome; the *colors* indicate that at least half of the genes were up-regulated (*black*), at least half of the genes down-regulated (*blue*), or more than half of the genes are unchanged (*orange*). If a gene was absent in one of the samples and present in another, it was regarded as more than a 2-fold change. A 2-fold level was chosen as this corresponded to one standard deviation in a double determination of ~1800 genes. Centromeres and heterochromatic regions were excluded from data analysis.

grade I and II, respectively, tumors 733 and 827 were staged as pT1 (invasive into submucosa), 733 was staged as solid, and 827 was staged as papillary, both grade III.

mRNA Preparation—Tissue biopsies, obtained fresh from surgery, were embedded immediately in a sodium-guanidinium thiocyanate solution and stored at −80 °C. Total RNA was isolated using the RNAzol B RNA isolation method (WAK-Chemie Medical GMBH). poly(A)$^+$ RNA was isolated by an oligo(dT) selection step (Oligotex mRNA kit; Qiagen).

cRNA Preparation—1 μg of mRNA was used as starting material. The first and second strand cDNA synthesis was performed using the SuperScript® choice system (Invitrogen) according to the manufacturer's instructions but using an oligo(dT) primer containing a T7 RNA polymerase binding site. Labeled cRNA was prepared using the MEGAscript® *in vitro* transcription kit (Ambion). Biotin-labeled CTP and UTP (Enzo) was used, together with unlabeled NTPs in the reaction. Following the *in vitro* transcription reaction, the unincorporated nucleotides were removed using RNeasy columns (Qiagen).

Array Hybridization and Scanning—Array hybridization and scanning was modified from a previous method (13). 10 μg of cRNA was fragmented at 94 °C for 35 min in buffer containing 40 mM Tris acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc. Prior to hybridization, the fragmented cRNA in a 6× SSPE-T hybridization buffer (1 M NaCl, 10 mM Tris, pH 7.6, 0.005% Triton), was heated to 95 °C for 5 min, subsequently cooled to 40 °C, and loaded onto the Affymetrix probe array cartridge. The probe array was then incubated for 16 h at 40 °C at constant rotation (60 rpm). The probe array was exposed to 10 washes in 6× SSPE-T at 25 °C followed by 4 washes in 0.5× SSPE-T at 50 °C. The biotinylated cRNA was stained with a streptavidin-phycoerythrin conjugate, 10 μg/ml (Molecular Probes) in 6× SSPE-T

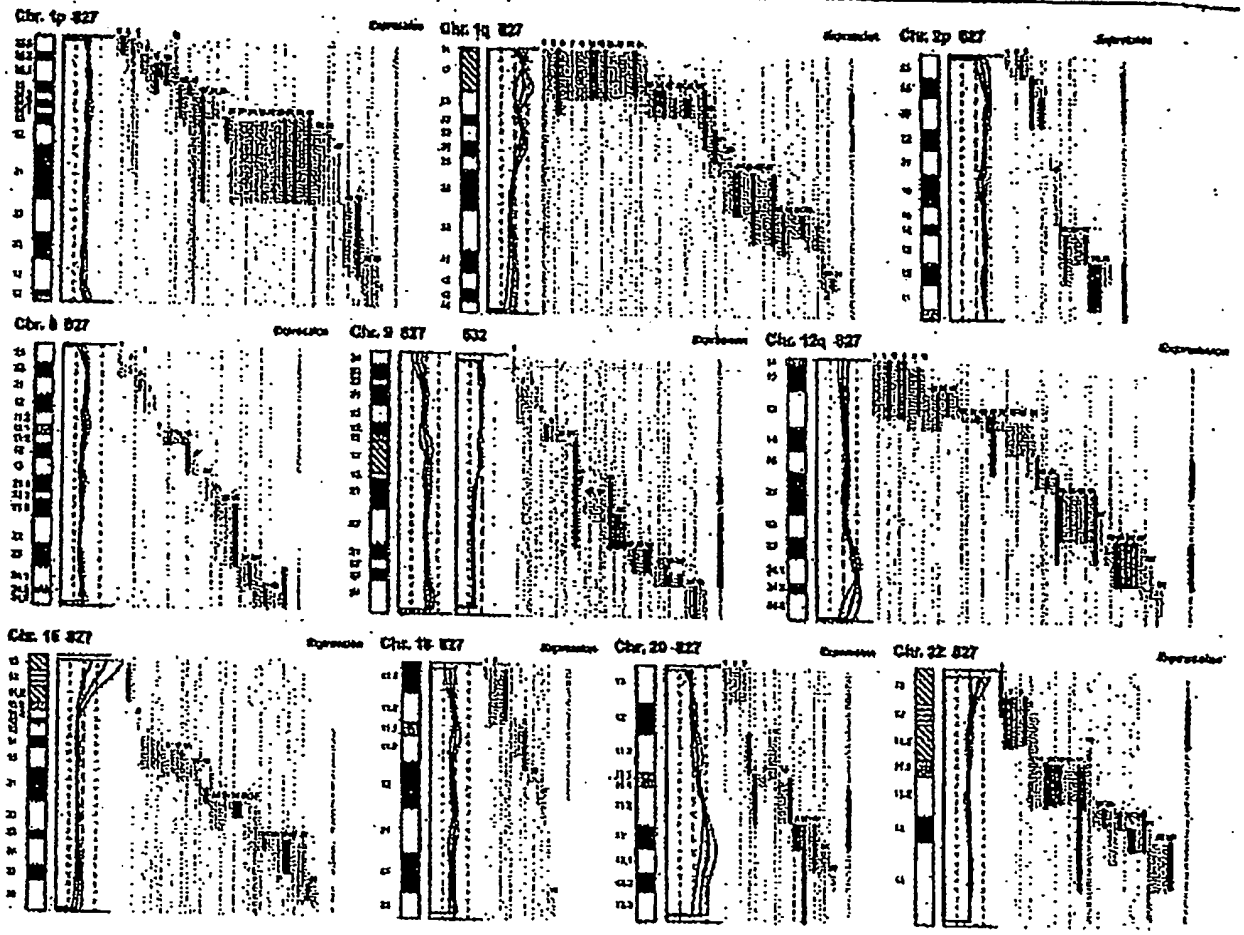

FIG. 1—*continued*

for 30 min at 25 °C followed by 10 washes in 6× SSPE-T at 25 °C. The probe arrays were scanned at 560 nm using a confocal laser scanning microscope (made for Affymetrix by Hewlett-Packard). The readings from the quantitative scanning were analyzed by Affymetrix gene expression analysis software.

Microsatellite Analysis—Microsatellite Analysis was performed as described previously (14). Microsatellites were selected by use of www.ncbi.nlm.nih.gov/genemap98, and primer sequences were obtained from the genome data base at www.gdb.org. DNA was extracted from tumor and blood and amplified by PCR in a volume of 20 μl for 35 cycles. The amplicons were denatured and electrophoresed for 3 h in an ABI Prism 377. Data were collected in the Gene Scan program for fragment analysis. Loss of heterozygosity was defined as less than 33% of one allele detected in tumor amplicons compared with blood.

Proteomic Analysis—TCCs were minced into small pieces and homogenized in a small glass homogenizer in 0.5 ml of lysis solution. Samples were stored at −20 °C until use. The procedure for 2D gel electrophoresis has been described in detail elsewhere (15, 16). Gels were stained with silver nitrate and/or Coomassie Brilliant Blue. Proteins were identified by a combination of procedures that included microsequencing, mass spectrometry, two-dimensional gel Western immunoblotting, and comparison with the master two-dimensional gel image of human keratinocyte proteins; see biobase.dk/cgi-bin/cells.

CGH—Hybridization of differentially labeled tumor and normal DNA to normal metaphase chromosomes was performed as described previously (10). Fluorescein-labeled tumor DNA (200 ng), Texas Red-labeled reference DNA (200 ng), and human Cot-1 DNA (20 μg) were denatured at 37 °C for 5 min and applied to denatured normal metaphase slides. Hybridization was at 37 °C for 2 days. After washing, the slides were counterstained with 0.15 μg/ml 4,6-diamidino-2-phenylindole in an anti-fade solution. A second hybridization was performed for all tumor samples using fluorescein-labeled reference DNA and Texas Red-labeled tumor DNA (inverse labeling) to confirm the aberrations detected during the initial hybridization. Each CGH experiment also included a normal control hybridization using fluorescein- and Texas Red-labeled normal DNA. Digital image analysis was used to identify chromosomal regions with abnormal fluorescence ratios, indicating regions of DNA gains and losses. The average green:red fluorescence intensity ratio profiles were calculated using four images of each chromosome (eight chromosomes total) with normalization of the green:red fluorescence intensity ratio for the entire metaphase and background correction. Chromosome identification was performed based on 4,6-diamidino-2-phenylindole banding patterns. Only images showing uniform high intensity fluorescence with minimal background staining were analyzed. All centromeres, p arms of acrocentric chromosomes, and heterochromatic regions were excluded from the analysis.

RESULTS

Comparative Genomic Hybridization—The CGH analysis identified a number of chromosomal gains and losses in the

TABLE I

Correlation between alterations detected by CGH and by expression monitoring

Top, CGH used as independent variable (if CGH alteration – what expression ratio was found); bottom, altered expression used as independent variable (if expression alteration – what CGH deviation was found).

CGH alterations	Tumor 733 vs. 335 Expression change clusters	Concordance	CGH alterations	Tumor 827 vs. 532 Expression change clusters	Concordance
13 Gain	10 Up-regulation 0 Down-regulation 3 No change	77%	10 Gain	8 Up-regulation 0 Down-regulation 2 No change	80%
10 Loss	1 Up-regulation 5 Down-regulation 4 No change	50%	12 Loss	3 Up-regulation 2 Down regulation 7 No change	17%

Expression change clusters	Tumor 733 vs. 335 CGH alterations	Concordance	Expression change clusters	Tumor 827 vs. 532 CGH alterations	Concordance
16 Up-regulation	11 Gain 2 Loss 3 No change	69%	17 Up-regulation	10 Gain 5 Loss 2 No change	59%
21 Down-regulation	1 Gain 8 Loss 12 No change	38%	9 Down-regulation	0 Gain 3 Loss 6 No change	33%
15 No change	3 Gain 3 Loss 9 No change	60%	21 No change	1 Gain 3 Loss 17 No change	81%

two invasive tumors (stage pT1, TCCs 733 and 827), whereas the two non-invasive papillomas (stage pTa, TCCs 335 and 532) showed only 9p–, 9q22-q33–, and X–, and 7+, 9q–, and Y–, respectively. Both invasive tumors showed changes (1q22-24+, 2q14.1-qter–, 3q12-q13.3–, 6q12-q22–, 9q34+, 11q12-q13+, 17+, and 20q11.2-q12+) that are typical for their disease stage, as well as additional alterations, some of which are shown in Fig. 1. Areas with gains and losses deviated from the normal copy number to some extent, and the average numerical deviation from normal was 0.4-fold in the case of TCC 733 and 0.3-fold for TCC 827. The largest changes, amounting to at least a doubling of chromosomal content, were observed at 1q23 in TCC 733 (Fig. 1*A*) and 20q12 in TCC 827 (Fig. 1*B*).

mRNA Expression in Relation to DNA Copy Number—The mRNA levels from the two invasive tumors (TCCs 827 and 733) were compared with the two non-invasive counterparts (TCCs 532 and 335). This was done in two separate experiments in which we compared TCCs 733 to 335 and 827 to 532, respectively, using two different scaling settings for the arrays to rule out scaling as a confounding parameter. Approximately 1,800 genes that yielded a signal on the arrays were searched in the Unigene and Genemap data bases for chromosomal location, and those with a known location (1096) were plotted as bars covering their purported locus. In that way it was possible to construct a graphic presentation of DNA copy number and relative mRNA levels along the individual chromosomes (Fig. 1).

For each mRNA a ratio was calculated between the level in the invasive *versus* the non-invasive counterpart. Bars, which represent chromosomal location of a gene, were color-coded according to the expression ratio, and only differences larger than 2-fold were regarded as informative (Fig. 1). The density of genes along the chromosomes varied, and areas containing only one gene were excluded from the calculations. The resolution of the CGH method is very low, and some of the outlier data may be because of the fact that the boundaries of the chromosomal aberrations are not known at high resolution.

Two sets of calculations were made from the data. For the first set we used CGH alterations as the independent variable and estimated the frequency of expression alterations in these chromosomal areas. In general, areas with a strong gain of chromosomal material contained a cluster of genes having increased mRNA expression. For example, both chromosomes 1q21-q25, 2p and 9q, showed a relative gain of more than 100% in DNA copy number that was accompanied by increased mRNA expression levels in the two tumor pairs (Fig. 1). In most cases, chromosomal gains detected by CGH were accompanied by an increased level of transcripts in both TCCs 733 (77%) and 827 (80%) (Table I, *top*). Chromosomal losses, on the other hand, were not accompanied by decreased expression in several cases, and were often registered as having unaltered RNA levels (Table I, *top*). The inability to detect RNA expression changes in these cases was not because of fewer genes mapping to the lost regions (data not shown).

In the second set of calculations we selected expression alterations above 2-fold as the independent variable and estimated the frequency of CGH alterations in these areas. As above, we found that increased transcript expression correlated with gain of chromosomal material (TCC 733, 69% and TCC 827, 59%), whereas reduced expression was often detected in areas with unaltered CGH ratios (Table I, *bottom*). Furthermore, as a control we looked at areas with no alter-

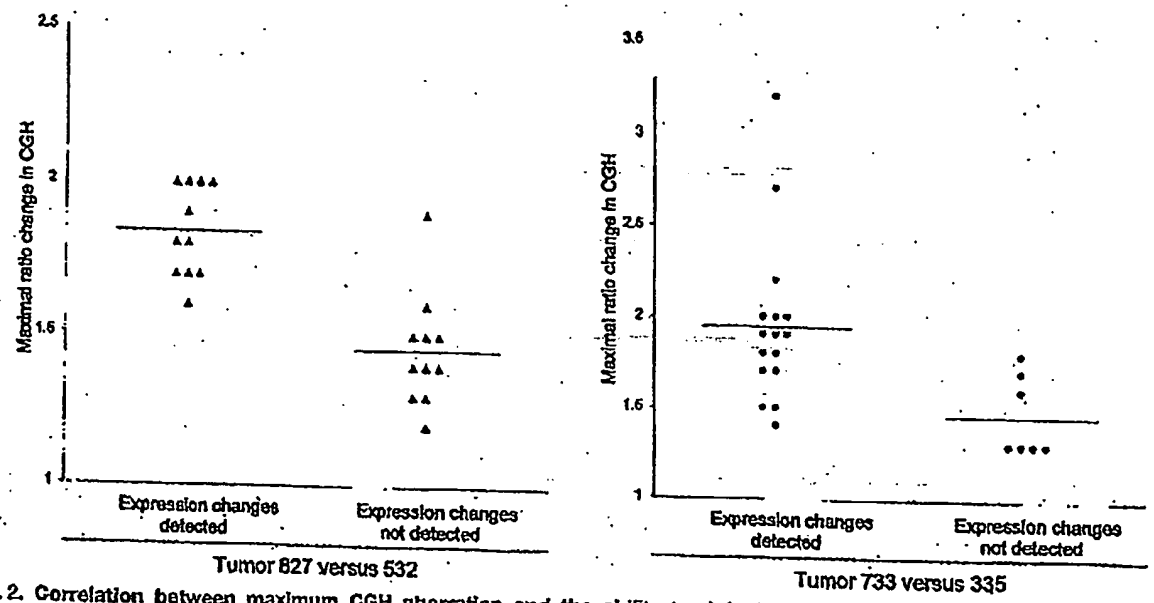

FIG. 2. Correlation between maximum CGH aberration and the ability to detect expression change by oligonucleotide array monitoring. The aberration is shown as a numerical -fold change in ratio between invasive tumors 827 (▲) and 733 (♦) and their non-invasive counterparts 532 and 335. The expression change was taken from the *Expression* line to the *right* in Fig. 1, which depicts the resulting expression change for a given chromosomal region. At least half of the mRNAs from a given region have to be either up- or down-regulated to be scored as an expression change. All chromosomal arms in which the CGH ratio plus or minus one standard deviation was outside the ratio value of one were included.

ation in expression. No alteration was detected by CGH in most of these areas (TCC 733, 60% and TCC 827, 81%; see Table I, *bottom*). Because the ability to observe reduced or increased mRNA expression clustering to a certain chromosomal area clearly reflected the extent of copy number changes, we plotted the maximum CGH aberrations in the regions showing CGH changes against the ability to detect a change in mRNA expression as monitored by the oligonucleotide arrays (Fig. 2). For both tumors TCC 733 ($p < 0.015$) and TCC 827 ($p < 0.00003$) a highly significant correlation was observed between the level of CGH ratio change (reflecting the DNA copy number) and alterations detected by the array based technology (Fig. 2). Similar data were obtained when areas with altered expression were used as independent variables. These areas correlated best with CGH when the CGH ratio deviated 1.6- to 2.0-fold (Table I, *bottom*) but mostly did not at lower CGH deviations. These data probably reflect that loss of an allele may only lead to a 50% reduction in expression level, which is at the cut-off point for detection of expression alterations. Gain of chromosomal material can occur to a much larger extent.

Microsatellite-based Detection of Minor Areas of Losses—In TCC 733, several chromosomal areas exhibiting DNA amplification were preceded or followed by areas with a normal CGH but reduced mRNA expression (see Fig. 1, TCC 733 chromosome 1q32, 2p21, and 7q21 and q32, 9q34, and 10q22). To determine whether these results were because of undetected loss of chromosomal material in these regions or because of other non-structural mechanisms regulating transcription, we examined two microsatellites positioned at chromosome 1q25–32 and two at chromosome 2p22. Loss of heterozygosity (LOH) was found at both 1q25 and at 2p22 indicating that minor deleted areas were not detected with the resolution of CGH (Fig. 3). Additionally, chromosome 2p in TCC 733 showed a CGH pattern of gain/no change/gain of DNA that correlated with transcript increase/decrease/increase. Thus, for the areas showing increased expression there was a correlation with the DNA copy number alterations (Fig. 1*A*). As indicated above, the mRNA decrease observed in the middle of the chromosomal gain was because of LOH, implying that one of the mechanisms for mRNA down-regulation may be regions that have undergone smaller losses of chromosomal material. However, this cannot be detected with the resolution of the CGH method.

In both TCC 733 and TCC 827, the telomeric end of chromosome 11p showed a normal ratio in the CGH analysis; however, clusters of five and three genes, respectively, lost their expression. Two microsatellites (D11S1760, D11S922) positioned close to MUC2, IGF2, and cathepsin D indicated LOH as the most likely mechanism behind the loss of expression (data not shown).

A reduced expression of mRNA observed in TCC 733 at chromosomes 3q24, 11p11, 12p12.2, 12q21.1, and 16q24 and in TCC 827 at chromosome 11p15.5, 12p11, 15q11.2, and 18q12 was also examined for chromosomal losses using microsatellites positioned as close as possible to the gene loci

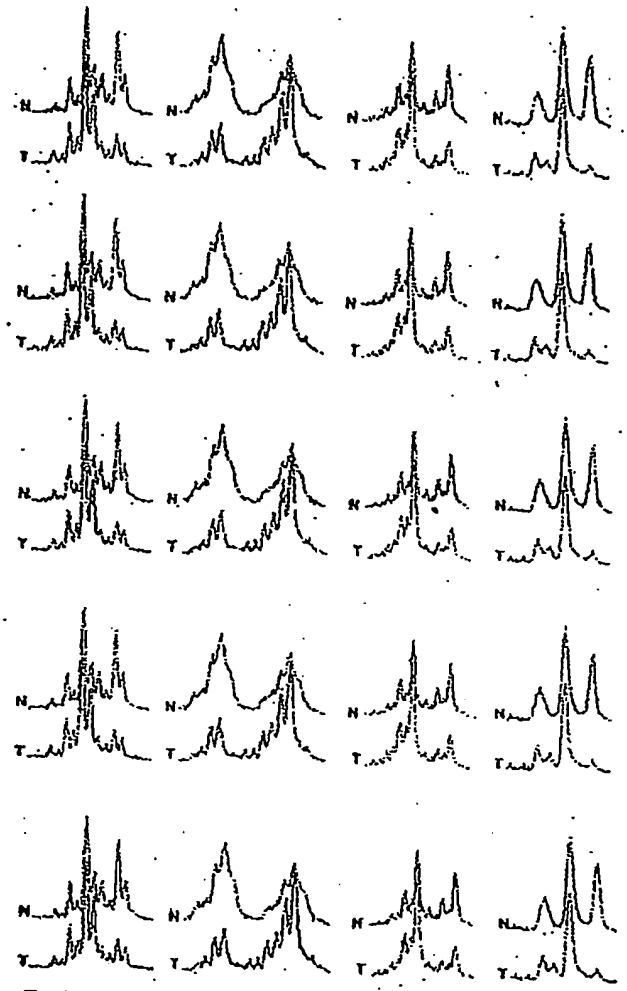

FIG. 3. Microsatellite analysis of loss of heterozygosity. Tumor 733 showing loss of heterozygosity at chromosome 1q25, detected (*a*) by D1S215 close to Hu class I histocompatibility antigen (gene number 38 in Fig. 1), (*b*) by D1S2735 close to cathepsin E (gene number 41 in Fig. 1), and (*c*) at chromosome 2p23 by D2S2251 close to general β-spectrin (gene number 11 on Fig. 1) and of (*d*) tumor 827 showing loss of heterozygosity at chromosome 18q12 by S18S1118 close to mitochondrial 3-oxoacyl-coenzyme A thiolase (gene number 12 in Fig. 1). The *upper curves* show the electropherogram obtained from normal DNA from leukocytes (*N*), and the *lower curves* show the electropherogram from tumor DNA (*T*). In all cases one allele is partially lost in the tumor amplicon.

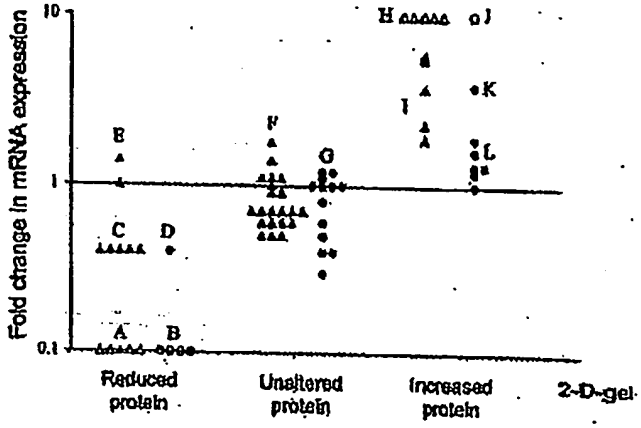

FIG. 4. Correlation between protein levels as judged by 2D-PAGE and transcript ratio. For comparison proteins were divided in three groups, unaltered in level or up- or down-regulated (*horizontal axis*). The mRNA ratio as determined by oligonucleotide arrays was plotted for each gene (*vertical axis*). ▲, mRNAs that were scored as present in both tumors used for the ratio calculation; △, mRNAs that were scored as absent in the invasive tumors (along *horizontal axis*) or as absent in non-invasive reference (*top* of figure). Two different scalings were used to exclude scaling as a confounder. TCCs 827 and 532 (▲△) were scaled with background suppression, and TCCs 733 and 335 (●○) were scaled without suppression. Both comparisons showed highly significant ($p < 0.005$) differences in mRNA ratios between the groups. Proteins shown were as follows: Group *A* (from *left*), phosphoglucomutase 1, glutathione transferase class μ number 4, fatty acid-binding protein homologue, cytokeratin 15, and cytokeratin 13; *B* (from *left*), fatty acid-binding protein homologue, 28-kDa heat shock protein, cytokeratin 13, and calcyclin; *C* (from *left*), α-enolase, hnRNP B1, 28-kDa heat shock protein, 14-3-3-ε, and pre-mRNA splicing factor; *D*, mesothelial keratin K7 (type II); *E* (from *top*), glutathione *S*-transferase-π and mesothelial keratin K7 (type II); *F* (from *top* and *left*), adenylyl cyclase-associated protein, E-cadherin, keratin 19, calgizzarin, phosphoglycerate mutase, annexin IV, cytoskeletal γ-actin, hnRNP A1, integral membrane protein calnexin (IP90), hnRNP H, brain-type clathrin light chain-a, hnRNP F, 70-kDa heat shock protein, heterogeneous nuclear ribonucleoprotein A/B, translationally controlled tumor protein, liver glyceraldehyde-3-phosphate dehydrogenase, keratin 8, aldehyde reductase, and Na,K-ATPase β-1 subunit; *G*, (from *top* and *left*), TCP20, calgizzarin, 70-kDa heat shock protein, calnexin, hnRNP H, cytokeratin 15, ATP synthase, keratin 19, triosephosphate isomerase, hnRNP F, liver glyceraldehyde-3-phosphatase dehydrogenase, glutathione *S*-transferase-π, and keratin 8; *H* (from *left*), plasma gelsolin, autoantigen calreticulin, thioredoxin, and NAD+-dependent 15 hydroxyprostaglandin dehydrogenase; *I* (from *top*), prolyl 4-hydroxylase β-subunit, cytokeratin 20, cytokeratin 17, prohibition, and fructose 1,6-biphosphatase; *J* annexin II; *K*, annexin IV; *L* (from *top* and *left*), 90-kDa heat shock protein, prolyl 4-hydroxylase β-subunit, α-enolase, GRP 78, cyclophilin, and cofilin.

showing reduced mRNA transcripts. Only the microsatellite positioned at 18q12 showed LOH (Fig. 3), suggesting that transcriptional down-regulation of genes in the other regions may be controlled by other mechanisms.

Relation between Changes in mRNA and Protein Levels—2D-PAGE analysis, in combination with Coomassie Brilliant Blue and/or silver staining, was carried out on all four tumors using fresh biopsy material. 40 well resolved abundant known proteins migrating in areas away from the edges of the pH gradient, and having a known chromosomal location, were selected for analysis in the TCC pair 827/532. Proteins were identified by a combination of methods (see "Experimental Procedures"). In general there was a highly significant correlation ($p < 0.005$) between mRNA and protein alterations (Fig. 4). Only one gene showed disagreement between transcript alteration and protein alteration. Except for a group of cyto-

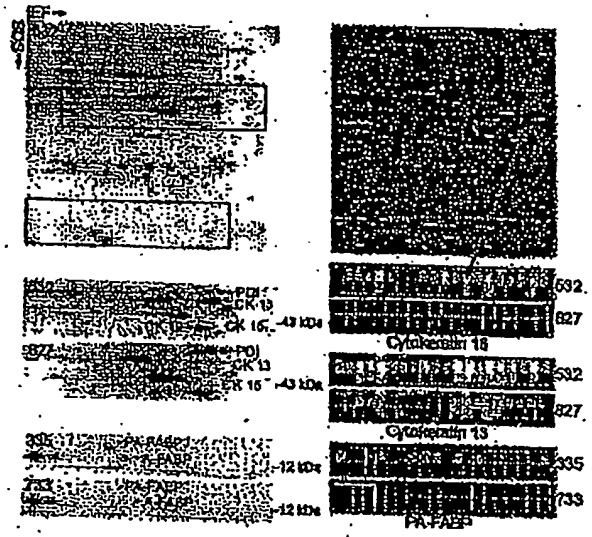

FIG. 5. **Comparison of protein and transcript levels in invasive and non-invasive TCCs.** The *upper part* of the figure shows a 2D gel (*left*) and the oligonucleotide array (*right*) of TCC 532. The *red rectangles* on the *upper gel* highlight the areas that are compared below. Identical areas of 2D gels of TCCs 532 and 827 are shown below. Clearly, cytokeratins 13 and 15 are strongly down-regulated in TCC 827 (*red annotation*). The tile on the array containing probes for cytokeratin 15 is enlarged *below* the array (*red arrow*) from TCC 532 and is compared with TCC 827. The *upper row* of *squares* in each tile corresponds to perfect match probes; the *lower row* corresponds to mismatch probes containing a mutation (used for correction for unspecific binding). Absence of signal is depicted as *black*, and the higher the signal the lighter the color. A high transcript level was detected in TCC 532 (6161 units) whereas a much lower level was detected in TCC 827 (absence of signals). For cytokeratin 13, a high transcript level was also present in TCC 532 (15659 units), and a much lower level was present in TCC 827 (623 units). The 2D gels at the *bottom* of the figure (*left*) show levels of PA-FABP and adipocyte-FABP in TCCs 335 and 733 (invasive), respectively. Both proteins are down-regulated in the invasive tumor. To the *right* we show the array tiles for the PA-FABP transcript. A medium transcript level was detected in the case of TCC 335 (1277 units) whereas very low levels were detected in TCC 733 (166 units). *IEF*, isoelectric focusing.

keratins encoded by genes on chromosome 17 (Fig. 5) the analyzed proteins did not belong to a particular family. 26 well focused proteins whose genes had a know chromosomal location were detected in TCCs 733 and 335, and of these 19 correlated ($p < 0.005$) with the mRNA changes detected using the arrays (Fig. 4). For example, PA-FABP was highly expressed in the non-invasive TCC 335 but lost in the invasive counterpart (TCC 733; see Fig. 5). The smaller number of proteins detected in both 733 and 335 was because of the smaller size of the biopsies that were available.

11 chromosomal regions where CGH showed aberrations that corresponded to the changes in transcript levels also showed corresponding changes in the protein level (Table II). These regions included genes that encode proteins that are found to be frequently altered in bladder cancer, namely cytokeratins 17 and 20, annexins II and IV, and the fatty acid-binding proteins PA-FABP and FBP1. Four of these proteins were encoded by genes in chromosome 17q, a frequently amplified chromosomal area in invasive bladder cancers.

DISCUSSION

Most human cancers have abnormal DNA content, having lost some chromosomal parts and gained others. The present study provides some evidence as to the effect of these gains and losses on gene expression in two pairs of non-invasive and invasive TCCs using high throughput expression arrays and proteomics. In combination with CGH. In general, the results showed that there is a clear individual regulation of the mRNA expression of single genes, which in some cases was superimposed by a DNA copy number effect. In most cases, genes located in chromosomal areas with gains often exhibited increased mRNA expression, whereas areas showing losses showed either no change or a reduced mRNA expression. The latter might be because of the fact that losses most often are restricted to loss of one allele, and the cut-off point for detection of expression alterations was a 2-fold change, thus being at the border of detection. In several cases, how-

TABLE II

Proteins whose expression level correlates with both mRNA and gene dose changes

Protein	Chromosomal location	Tumor TCC	CGH alteration	Transcript alteration[a]	Protein alteration
Annexin II	1q21	733	Gain	Abs to Pres[a]	Increase
Annexin IV	2p13	733	Gain	3.9-Fold up	Increase
Cytokeratin 17	17q12-q21	827	Gain	3.8-Fold up	Increase
Cytokeratin 20	17q21.1	827	Gain	5.6-Fold up	Increase
(PA-)FABP	8q21.2	827	Loss	10-Fold down	Decrease
FBP1	9q22	827	Gain	2.3-Fold up	Increase
Plasma gelsolin	9q31	827	Gain	Abs to Pres	Increase
Heat shock protein 28	15q12-q13	827	Loss	2.5-Fold up	Decrease
Prohibitin	17q21	827/733	Gain	3.7-/2.5-Fold up[b]	Increase
Prolyl-4-hydroxyl	17q25	827/733	Gain	5.7-/1.6-Fold up	Increase
hnRNPB1	7p15	827	Loss	2.5-Fold down	Decrease

[a] Abs, absent; Pres, present.

[b] In cases where the corresponding alterations were found in both TCCs 827 and 733 these are shown as 827/733.

ever, an increase or decrease in DNA copy number was associated with *de novo* occurrence or complete loss of transcript, respectively. Some of these transcripts could not be detected in the non-invasive tumor but were present at relatively high levels in areas with DNA amplifications in the invasive tumors (*e.g.* in TCC 733 transcript from cellular ligand of annexin II gene (chromosome 1q21) from absent to 2670 arbitrary units; in TCC 827 transcript from small proline-rich protein 1 gene (chromosome 1q12-q21.1) from absent to 1326 arbitrary units). It may be anticipated from these data that significant clustering of genes with an increased expression to a certain chromosomal area indicates an increased likelihood of gain of chromosomal material in this area.

Considering the many possible regulatory mechanisms acting at the level of transcription, it seems striking that the gene dose effects were so clearly detectable in gained areas. One hypothetical explanation may lie in the loss of controlled methylation in tumor cells (17–19). Thus, it may be possible that in chromosomes with increased DNA copy numbers two or more alleles could be demethylated simultaneously leading to a higher transcription level, whereas in chromosomes with losses the remaining allele could be partly methylated, turning off the process (20, 21). A recent report has documented a ploidy regulation of gene expression in yeast, but in this case all the genes were present in the same ratio (22), a situation that is not analogous to that of cancer cells, which show marked chromosomal aberrations, as well as gene dosage effects.

Several CGH studies of bladder cancer have shown that some chromosomal aberrations are common at certain stages of disease progression, often occurring in more than 1 of 3 tumors. In pTa tumors, these include 9p−, 9q−, 1q+, Y− (2, 6), and in pT1 tumors, 2q−, 11p−, 11q−, 1q+, 5p+, 8q+, 17q+, and 20q+ (2–4, 6, 7). The pTa tumors studied here showed similar aberrations such as 9p− and 9q22-q33− and 9q− and Y−, respectively. Likewise, the two minimal invasive pT1 tumors showed aberrations that are commonly seen at that stage, and TCC 827 had a remarkable resemblance to the commonly seen pattern of losses and gains, such as 1q22–24 amplification (seen in both tumors), 11q14-q22 loss, the latter often linked to 17 q+ (both tumors), and 1q+ and 9p−, often linked to 20q+ and 11 q13+ (both tumors) (7–9). These observations indicate that the pairs of tumors used in this study exhibit chromosomal changes observed in many tumors, and therefore the findings could be of general importance for bladder cancer.

Considering that the mapping resolution of CGH is of about 20 megabases it is only possible to get a crude picture of chromosomal instability using this technique. Occasionally, we observed reduced transcript levels close to or inside regions with increased copy numbers. Analysis of these regions by positioning heterozygous microsatellites as close as possible to the locus showing reduced gene expression revealed loss of heterozygosity in several cases. It seems likely that multiple and different events occur along each chromosomal arm and that the use of cDNA microarrays for analysis of DNA copy number changes will reach a resolution that can resolve these changes, as has recently been proposed (2). The outlier data were not more frequent at the boundaries of the CGH aberrations. At present we do not know the mechanism behind chromosomal aneuploidy and cannot predict whether chromosomal gains will be transcribed to a larger extent than the two native alleles. A mechanism as genetic imprinting has an impact on the expression level in normal cells and is often reduced in tumors. However, the relation between imprinting and gain of chromosomal material is not known.

We regard it as a strength of this investigation that we were able to compare invasive tumors to benign tumors rather than to normal urothelium, as the tumors studied were biologically very close and probably may represent successive steps in the progression of bladder cancer. Despite the limited amount of fresh tissue available it was possible to apply three different state of the art methods. The observed correlation between DNA copy number and mRNA expression is remarkable when one considers that different pieces of the tumor biopsies were used for the different sets of experiments. This indicate that bladder tumors are relatively homogenous, a notion recently supported by CGH and LOH data that showed a remarkable similarity even between tumors and distant metastasis (10, 23).

In the few cases analyzed, mRNA and protein levels showed a striking correspondence although in some cases we found discrepancies that may be attributed to translational regulation, post-translational processing, protein degradation, or a combination of these. Some transcripts belong to undertranslated mRNA pools, which are associated with few translationally inactive ribosomes; these pools, however, seem to be rare (24). Protein degradation, for example, may be very important in the case of polypeptides with a short half-life (*e.g.* signaling proteins). A poor correlation between mRNA and protein levels was found in liver cells as determined by arrays and 2D-PAGE (25), and a moderate correlation was recently reported by Ideker *et al.* (26) in yeast.

Interestingly, our study revealed a much better correlation between gained chromosomal areas and increased mRNA levels than between loss of chromosomal areas and reduced mRNA levels. In general, the level of CGH change determined the ability to detect a change in transcript. One possible explanation could be that by losing one allele the change in mRNA level is not so dramatic as compared with gain of material, which can be rather unlimited and may lead to a severalfold increase in gene copy number resulting in a much higher impact on transcript level. The latter would be much easier to detect on the expression arrays as the cut-off point was placed at a 2-fold level so as not to be biased by noise on the array. Construction of arrays with a better signal to noise ratio may in the future allow detection of lesser than 2-fold alterations in transcript levels, a feature that may facilitate the analysis of the effect of loss of chromosomal areas on transcript levels.

In eleven cases we found a significant correlation between DNA copy number, mRNA expression, and protein level. Four of these proteins were encoded by genes located at a frequently amplified area in chromosome 17q. Whether DNA copy number is one of the mechanisms behind alteration of these eleven proteins is at present unknown and will have to be proved by other methods using a larger number of samples. One factor making such studies complicated is the large extent of protein modification that occurs after translation, requiring immunoidentification and/or mass spectrometry to correctly identify the proteins in the gels.

In conclusion, the results presented in this study exemplify the large body of knowledge that may be possible to gather in the future by combining state of the art techniques that follow the pathway from DNA to protein (26). Here, we used a traditional chromosomal CGH method, but in the future high resolution CGH based on microarrays with many thousand radiation hybrid-mapped genes will increase the resolution and information derived from these types of experiments (2). Combined with expression arrays analyzing transcripts derived from genes with known locations, and 2D gel analysis to obtain information at the post-translational level, a clearer and more developed understanding of the tumor genome will be forthcoming.

Acknowledgments—We thank Mie Madsen, Hanne Steen, Inge Lis Thorsen, Hans Lund, Vikolaj Ørntoft, and Lynn Bjerke for technical help and Thomas Gingeras, Christine Harrington, and Morten Østergaard for valuable discussions.

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EXHIBIT 3

214: Urol Res. 2000 Oct;28(5):308-15.

Expression of cadherins and catenins in paired tumor and non-neoplastic primary prostate cultures and corresponding prostatectomy specimens.

Wang J, Krill D, Torbenson M, Wang Q, Bisceglia M, Stoner J, Thomas A, DeFlavia P, Dhir R, Becich MJ.

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Cadherins are a family of transmembrane proteins that play a crucial role in cell differentiation, cell migration, and intercellular adhesion. Cadherins are associated with catenins through their highly conserved cytoplasmic domain. Down-regulation of E-cadherin protein has been shown in various human cancers. This study examined the expression of cadherins and associated catenins at the mRNA level. Paired tumor and nonneoplastic primary prostate cultures were obtained from surgical specimens. Quantitative multiplex fluorescence reverse transcriptase-polymerase chain reaction (QMF RT-PCR) and quantitative analysis were performed and correlated with immunostain results. Six of seven cases of neoplastic cultures showed moderately-to-markedly decreased levels of E-cadherin and P-cadherin mRNA. Similar losses of alpha-catenin and beta-catenin mRNA were also observed. The results of QMF RT-PCR showed good correlation with the results of immunohistochemical studies based on corresponding formalin-fixed sections. In conclusion, this paper presents a coordinated down-regulation in the expression of E-cadherin and associated catenins at the mRNA and protein level in most of the cases studied. This down-regulation may play an important role in the pathogenesis of prostate cancer.

PMID: 11127708 [PubMed - indexed for MEDLINE]

EXHIBIT 4

93: Int J Cancer. 2003 Oct 10;106(6):848-55.

Vascular endothelial growth factor expression correlates with matrix metalloproteinases MT1-MMP, MMP-2 and MMP-9 in human glioblastomas.

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Vascular endothelial growth factor (VEGF) is the major endothelial mitogen in central nervous system neoplasms and it is expressed in 64-95% of glioblastomas (GBMs). Tumour cells are the main source of VEGF in GBMs whereas VEGF receptors (VEGFR-1, its soluble form sVEGFR-1, VEGFR-2 and neuropilin-1) are expressed predominantly by endothelial cells. Infiltrating tumour cells and newly-formed capillaries progress through the extracellular matrix by local proteolysis involving matrix metalloproteinases (MMPs). Recent studies have shown that VEGF expression and bioavailability can be modulated by MMPs. We reported previously that the expression of MT1-MMP in human breast cancer cells was associated with an enhanced VEGF expression. We used quantitative RT-PCR, Western blot, gelatin zymography and immunohistochemistry to study the expression of VEGF, VEGFR-1, VEGFR-2, sVEGFR-1, neuropilin-1, MT1-MMP, MMP-2, MMP-9 and TIMP-2 in 20 human GBMs and 5 normal brains. The expression of these MMPs was markedly increased in most GBMs with excellent correlation between mRNA and protein levels; activated forms of MMP-2 and MMP-9 were present in 8/18 and 7/18 of GBMs. A majority of GBMs (17/20) also expressed high levels of VEGF, as previously reported, with strong correlation between VEGF and MT1-MMP gene expression levels, and double immunostaining showed that VEGF and MT1-MMP peptides co-localize in tumour and endothelial cells. Our results suggest that the interplay between metalloproteinases and VEGF previously described in experimental tumours may also be operative in human GBMs. Because of its dual ability to activate MMP-2 and to up-regulate VEGF, MT1-MMP might be of central importance in the growth of GBMs and represent an interesting target for anti-cancer treatments. Copyright 2003 Wiley-Liss, Inc.

PMID: 12918061 [PubMed - indexed for MEDLINE]

EXHIBIT 5

89: Leuk Lymphoma. 2003 Aug;44(8):1385-94. Related Articles, Links

Real-time quantitative RT-PCR of cyclin D1 mRNA in mantle cell lymphoma: comparison with FISH and immunohistochemistry.

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Presence of the balanced translocation t(11;14)(q13;q32) and the consequent overexpression of cyclin D1 found in mantle cell lymphoma (MCL) has been shown to be of important diagnostic value. Although many molecular and immunohistochemical approaches have been applied to analyze cyclin D1 status, correlative studies to compare different methods for the diagnosis of MCL are lacking. In this study, we examined 39 archived paraffin specimens from patients diagnosed with a variety of lymphoproliferative diseases including nine cases meeting morphologic and immunophenotypic criteria for MCL by: (1) real-time quantitative RT-PCR to evaluate cyclin D1 mRNA expression; (2) dual fluorescence in situ hybridization (FISH) to evaluate the t(11;14) translocation in interphase nuclei; and (3) tissue array immunohistochemistry to evaluate the cyclin D1 protein level. Among the nine cases of possible MCL, seven cases showed overexpression of cyclin D1 mRNA (cyclin D1 positive MCL) and two cases showed no cyclin D1 mRNA increase (cyclin D1 negative "MCL-like"). In six of seven cyclin D1 positive cases, the t(11;14) translocation was demonstrated by FISH analysis; in one case FISH was unsuccessful. Six of the seven cyclin D1 mRNA overexpressing cases showed increased cyclin D1 protein on tissue array immunohistochemistry; one was technically suboptimal. Among the two cyclin D1 negative MCL-like cases, FISH confirmed the absence of the t(11;14) translocation in both cases. All other lymphoproliferative diseases studied were found to have low or no cyclin D1 mRNA expression and were easily distinguishable from the cyclin D1 overexpressing MCLs by all three techniques. In addition, to confirming the need to assess cyclin D1 status, as well as, morphology and immunophenotyping to establish the diagnosis of MCL, this study demonstrates good correlation and comparability between measure of cyclin D1 mRNA, the 11;14 translocation and cyclin D1 protein.

Publication Types:

- Evaluation Studies

PMID: 12952233 [PubMed - indexed for MEDLINE]

EXHIBIT 6

7. 8: Int J Biochem Cell Biol. 2005 Oct;37(10):2196-206. Epub 2004 Dec

Related Articles, Links

ELSEVIER FULL TEXT ARTICLE

Increased expression of proteasome subunits in skeletal muscle of cancer patients with weight loss.

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Atrophy of skeletal muscle is common in patients with cancer and results in increased morbidity and mortality. In order to design effective therapy the mechanism by which this occurs needs to be elucidated. Most studies suggest that the ubiquitin-proteasome proteolytic pathway is most important in intracellular proteolysis, although there have been no reports on the activity of this pathway in patients with different extents of weight loss. In this report the expression of the ubiquitin-proteasome pathway in rectus abdominis muscle has been determined in cancer patients with weight loss of 0-34% using a competitive reverse transcriptase polymerase chain reaction to measure expression of mRNA for proteasome subunits C2 and C5, while protein expression has been determined by western blotting. Overall, both C2 and C5 gene expression was increased by about three-fold in skeletal muscle of cachectic cancer patients (average weight loss 14.5+/-2.5%), compared with that in patients without weight loss, with or without cancer. The level of gene expression was dependent on the amount of weight loss, increasing maximally for both proteasome subunits in patients with weight loss of 12-19%. Further increases in weight loss reduced expression of mRNA for both proteasome subunits, although it was still elevated in comparison with patients with no weight loss. There was no evidence for an increase in expression at weight losses less than 10%. There was a good correlation between expression of proteasome 20Salpha subunits, detected by western blotting, and C2 and C5 mRNA, showing that increased gene expression resulted in increased protein synthesis. Expression of the ubiquitin conjugating enzyme, E2(14k), with weight loss followed a similar pattern to that of proteasome subunits. These results suggest variations in the expression of key components of the ubiquitin-proteasome pathway with weight loss of cancer patients, and suggest that another mechanism of protein degradation must be operative for patients with weight loss less than 10%.

PMID: 16125116 [PubMed - in process]

EXHIBIT 7

269: Am J Pathol. 1999 Sep;155(3):815-22. Related Articles, Links

Id-1 and Id-2 are overexpressed in pancreatic cancer and in dysplastic lesions in chronic pancreatitis.

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Id proteins antagonize basic helix-loop-helix proteins, inhibit differentiation, and enhance cell proliferation. In this study we compared the expression of Id-1, Id-2, and Id-3 in the normal pancreas, in pancreatic cancer, and in chronic pancreatitis (CP). Northern blot analysis demonstrated that all three Id mRNA species were expressed at high levels in pancreatic cancer samples by comparison with normal or CP samples. Pancreatic cancer cell lines frequently coexpressed all three Ids, exhibiting a good correlation between Id mRNA and protein levels, as determined by immunoblotting with highly specific anti-Id antibodies. Immunohistochemistry using these antibodies demonstrated the presence of faint Id-1 and Id-2 immunostaining in pancreatic ductal cells in the normal pancreas, whereas Id-3 immunoreactivity ranged from weak to strong. In the cancer tissues, many of the cancer cells exhibited abundant Id-1, Id-2, and Id-3 immunoreactivity. Scoring on the basis of percentage of positive cells and intensity of immunostaining indicated that Id-1 and Id-2 were increased significantly in the cancer cells by comparison with the respective controls. Mild to moderate Id immunoreactivity was also seen in the ductal cells in the CP-like areas adjacent to these cells and in the ductal cells of small and interlobular ducts in CP. In contrast, in dysplastic and atypical papillary ducts in CP, Id-1 and Id-2 immunoreactivity was as significantly elevated as in the cancer cells. These findings suggest that increased Id expression may be associated with enhanced proliferative potential of pancreatic cancer cells and of proliferating or dysplastic ductal cells in CP.

PMID: 10487839 [PubMed - indexed for MEDLINE]

EXHIBIT 8

283: Neurosci Lett. 1999 Apr 23;265(3):191-4.

ELSEVIER
FULL TEXT ARTICLE

Alterations in neuropeptide Y levels and Y1 binding sites in the Flinders Sensitive Line rats, a genetic animal model of depression.

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Previously, we observed specific alterations of neuropeptide Y (NPY) and Y1 receptor mRNA expression in discrete regions of the Flinders Sensitive Line rats (FSL), an animal model of depression. In order to clarify the correlation between mRNA expression and protein content, radioimmunoassay and receptor autoradiography were currently performed. In the FSL rats, NPY-like immunoreactivity (NPY-LI) was decreased in the hippocampal CA region, while Y1 binding sites were increased; NPY-LI was increased in the arcuate nucleus. Fluoxetine treatment elevated NPY-LI in the arcuate and anterior cingulate cortex and increased Y1 binding sites in the medial amygdala and occipital cortex in both strains. No differences were found regarding the Y2 binding sites. The results demonstrate a good correlation between NPY peptide and mRNA expression, and sustain the possible involvement of NPY and Y1 receptors in depression.

PMID: 10327163 [PubMed - indexed for MEDLINE]

EXHIBIT 9

271: Biol Reprod. 1999 Sep;61(3):776-84.

Follicle-stimulating hormone receptor and its messenger ribonucleic acid are present in the bovine cervix and can regulate cervical prostanoid synthesis.

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The hypothesis that FSH regulates the bovine cervical prostaglandin E(2) (PGE(2)) synthesis that is known to be associated with cervical relaxation and opening at the time of estrus was investigated. Cervical tissue from pre-estrous/estrous, luteal, and postovulatory cows were examined for 1) the presence of bovine (b) FSH receptor (R) and its corresponding mRNA and 2) the effect of FSH on the PGE(2) regulatory pathway in vitro. The presence of bFSHR mRNA in the cervix (maximal during pre-estrus/estrus) was demonstrated by the expression of a reverse transcription (RT) polymerase chain reaction (PCR) product (384 base pairs) specific for bFSHR mRNA and sequencing. Northern blotting revealed three transcripts (2.5, 3.3, and 3.8 kilobases [kb]) in cervix from pre-estrous/estrous cows. The level of FSHR (75 kDa) was significantly higher ($p < 0.01$) in Western blots of pre-estrous/estrous cervix than in other cervical tissues. There was a good correlation between the 75-kDa protein expression and its corresponding transcript of 2.55 kb throughout the estrous cycle as described by Northern blot analysis as well as RT-PCR. Incubation of FSH (10 ng/ml) with pre-estrous/estrous cervix resulted in a 3-fold increase in the expression of FSHR and a 2-fold increase in both G protein (alpha(s)) and cyclooxygenase II. FSH (5-20 ng/ml) significantly increased ($p < 0.01$) cAMP, inositol phosphate ($p < 0.01$), and PGE(2) ($p < 0.01$) production by pre-estrous/estrous cervix but not by cervix at the other stages. We conclude that bovine cervix at the time of the peripheral plasma FSH peak (pre-estrus/estrus) contains high levels of FSHR and responds to FSH by increasing the PGE(2) production responsible for cervical relaxation at estrus.

PMID: 10456856 [PubMed - indexed for MEDLINE]

EXHIBIT 10

232: J Urol. 2000 Sep;164(3 Pt 2):1026-30.

LWWonline
Lippincott Williams & Wilkins

The decompensated detrusor III: impact of bladder outlet obstruction on sarcoplasmic endoplasmic reticulum protein and gene expression.

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PURPOSE: Regulation of calcium ion homeostasis has a significant role in smooth muscle contractility. The sarcoplasmic endoplasmic reticulum, calcium, magnesium, adenosine triphosphatase (SERCA) is a regulatory ion pump that may have a role in the functional outcome after outlet obstruction. We investigate what correlation if any existed between SERCA protein and gene expression, and the contractile properties in the same bladder. MATERIALS AND METHODS: Standardized partial bladder outlet obstructions were created in adult New Zealand white rabbits, which were divided into control, sham operated and obstructed groups. Muscle strip studies subcategorized the obstructed group into compensated (force greater than 50% of control) and decompensated (force less than 50% of control). Microsomal membrane and total RNA fractions were prepared from the same bladder tissue. Membrane proteins were used for Western blot analysis using a SERCA specific monoclonal antibody, and total RNA was assessed with Northern blot analysis. RESULTS: The relative intensities of signals for the Western and Northern blots demonstrated a strong correlation between protein and gene expression. Furthermore there was a strong association between the loss of SERCA messenger RNA and protein expression and loss of bladder function. CONCLUSIONS: Bladder contractility after outlet obstruction is influenced in part by smooth muscle cell ability to maintain calcium homeostasis via SERCA. The loss of SERCA protein expression is mediated by down-regulation in gene expression in the same bladder. These data suggest that smooth muscle ion pump gene expression is in part mechanically (pressure work) regulated.

PMID: 10958733 [PubMed - indexed for MEDLINE]

EXHIBIT 11

141: Zhonghua Jie He He Hu Xi Za Zhi. 2002 Jun;25(6):337-40. Related Articles, Links

[The pathogenic role of macrophage migration inhibitory factor in acute respiratory distress syndrome]

[Article in Chinese]

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OBJECTIVE To investigate the expression and pathogenic role of macrophage migration inhibitory factor(MIF) in human acute respiratory distress syndrome(ARDS)
METHODS The serum level of MIF in ARDS patients and normal persons were measured by ELISA method. Peripheral blood mononuclear cell (PBMC) MIF expression was determined by flow- cytometry. The expression of MIF mRNA and protein in the lung tissues were detected by using double immuno histochemistry labeling and in situ hybridization. RESULTS The serum level of MIF increased significantly in ARDS patients as compared with normal persons ($P < 0.01$). The percentage of PBMC MIF expression was higher in ARDS patients than in normal controls ($P < 0.01$). In situ hybridization and immunohistochemistry showed undetectable or weak MIF mRNA and protein expression in normal lungs. In contrast, there was marked upregulation of MIF mRNA and protein expression in the ARDS lungs. In ARDS macro phages infiltrated the alveolar space and interstitium, most of which also expressed MIF. Infiltrating macrophages were almost restricted to the areas of severe tissue damage. The MIF expression level showed a strong correlation with the number of infiltrating macrophages.
CONCLUSIONS The serum level of MIF and PBMC MIF expression increased in ARDS patients with enhanced pulmonary MIF expression and macrophage infiltration, which suggests that MIF plays a pivotal role in the pathogenesis of ARDS.

PMID: 12126556 [PubMed - indexed for MEDLINE]

EXHIBIT 12

1:Int J Oncol. 2005 Nov;27(5):1257-63. Related Articles, Links

Expression of human telomerase reverse transcriptase gene and protein, and of estrogen and progesterone receptors, in breast tumors: Preliminary data from neo-adjuvant chemotherapy.

Kammori M, Izumiyama N, Hashimoto M, Nakamura K, Okano T, Kurabayashi R, Naoki H, Honma N, Ogawa T, Kaminishi M, Takubo K.

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Human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, is very closely associated with telomerase activity. Telomerase has been implicated in cellular immortalization and carcinogenesis. in situ detection of hTERT will aid in determining the localization of telomerase-positive cells. The aim of this study was to detect expression of hTERT mRNA, hTERT protein, estrogen receptor (ER) and progesterone receptor (PR) in paraffin-embedded breast tissue samples and to investigate the relationship between hTERT expression and various clinicopathological parameters in breast tumorigenesis. We used in situ hybridization (ISH) to examine hTERT gene expression, and immunohistochemistry (IHC) to examine expression of hTERT protein, ER and PR, in breast tissues including 64 adenocarcinomas, 2 phyllode tumors and their adjacent normal breast tissues. hTERT gene expression was detected by ISH in 56 (88%) carcinomas, but in neither of the 2 phyllode tumors. hTERT protein expression was detected by IHC in 52 (81%) carcinomas, but in neither of the 2 phyllode tumors. Moreover, ER and PR were expressed in 42 (66%) and 42 (66%) carcinomas, respectively, and in neither of the 2 phyllode tumors. In 4 cases of breast carcinoma that strongly expressed hTERT gene and protein before treatment, neo-adjuvant chemotherapy led to disappearance of gene and protein expression in all cases. There was a strong correlation between detection of hTERT gene expression by ISH and of hTERT protein by ICH in tissue specimens from breast tumors. These results suggest that detection of hTERT protein by ICH can be used to distinguish breast cancers as a potential diagnostic and therapeutic marker.

PMID: 16211220 [PubMed - in process]

EXHIBIT 13

266: Mol Cell Biol. 1999 Nov;19(11):7357-68. Related Articles, Links

A sampling of the yeast proteome.

Futcher B, Latter GI, Monardo P, McLaughlin CS, Garrels JI.

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In this study, we examined yeast proteins by two-dimensional (2D) gel electrophoresis and gathered quantitative information from about 1,400 spots. We found that there is an enormous range of protein abundance and, for identified spots, a good correlation between protein abundance, mRNA abundance, and codon bias. For each molecule of well-translated mRNA, there were about 4,000 molecules of protein. The relative abundance of proteins was measured in glucose and ethanol media. Protein turnover was examined and found to be insignificant for abundant proteins. Some phosphoproteins were identified. The behavior of proteins in differential centrifugation experiments was examined. Such experiments with 2D gels can give a global view of the yeast proteome.

PMID: 10523624 [PubMed - indexed for MEDLINE]

EXHIBIT 14

304: J Biol Chem. 1998 Aug 14;273(33):21161-8.

Overexpression of a DEAD box protein (DDX1) in neuroblastoma and retinoblastoma cell lines.

Godbout R, Packer M, Bie W.

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The DEAD box gene, DDX1, is a putative RNA helicase that is co-amplified with MYCN in a subset of retinoblastoma (RB) and neuroblastoma (NB) tumors and cell lines. Although gene amplification usually involves hundreds to thousands of kilobase pairs of DNA, a number of studies suggest that co-amplified genes are only overexpressed if they provide a selective advantage to the cells in which they are amplified. Here, we further characterize DDX1 by identifying its putative transcription and translation initiation sites. We analyze DDX1 protein levels in MYCN/DDX1-amplified NB and RB cell lines using polyclonal antibodies specific to DDX1 and show that there is a good correlation with DDX1 gene copy number, DDX1 transcript levels, and DDX1 protein levels in all cell lines studied. DDX1 protein is found in both the nucleus and cytoplasm of DDX1-amplified lines but is localized primarily to the nucleus of nonamplified cells. Our results indicate that DDX1 may be involved in either the formation or progression of a subset of NB and RB tumors and suggest that DDX1 normally plays a role in the metabolism of RNAs located in the nucleus of the cell.

PMID: 9694872 [PubMed - indexed for MEDLINE]

EXHIBIT 15

150: Virchows Arch. 2002 May;440(5):461-75. Epub 2002 Mar 23. Related Articles, Links
SpringerLink FULL-TEXT ARTICLE

Expression of somatostatin receptor types 1-5 in 81 cases of gastrointestinal and pancreatic endocrine tumors. A correlative immunohistochemical and reverse-transcriptase polymerase chain reaction analysis.

Papotti M, Bongiovanni M, Volante M, Allia E, Landolfi S, Helboe L, Schindler M, Cole SL, Bussolati G.

Department of Biomedical Sciences and Oncology, University of Turin, Via Santena 7, 10126 Turin, Italy. mauro.papotti@unito.it

Somatostatin receptors (SSTRs) have been extensively mapped in human tumors by means of autoradiography, reverse-transcriptase polymerase chain reaction (RT-PCR), in situ hybridization (ISH) and immunohistochemistry (IHC). We analyzed the SSTR type 1-5 expression by means of RT-PCR and/or IHC in a series of 81 functioning and non-functioning gastroenteropancreatic (GEP) endocrine tumors and related normal tissues. Moreover, we compared the results with clinical, pathological and hormonal features. Forty-six cases (13 intestinal and 33 pancreatic) were studied for SSTR 1-5 expression using RT-PCR, IHC with antibodies to SSTR types 2, 3, 5 and ISH for SSTR2 mRNA. The vast majority of tumors expressed SSTR types 1, 2, 3 and 5, while SSTR4 was detected in a small minority. Due to the good correlation between RT-PCR and IHC data on SSTR types 2, 3, and 5, thirty-five additional GEP endocrine tumors were studied with IHC alone. Pancreatic insulinomas had an heterogeneous SSTR expression, while 100% of somatostatinomas expressed SSTR5 and 100% gastrinomas and glucagonomas expressed SSTR2. Pre-operative biopsy material showed an overlapping immunoreactivity with that of surgical specimens, suggesting that the SSTR status can be detected in the diagnostic work-up. It is concluded that SSTRs 1-5 are heterogeneously expressed in GEP endocrine tumors and that IHC is a reliable tool to detect SSTR types 2, 3 and 5 in surgical and biopsy specimens.

PMID: 12021920 [PubMed - indexed for MEDLINE]

EXHIBIT 16

114: Eur J Cancer. 2003 Mar;39(5):691-7.

Expression of deoxycytidine kinase in leukaemic cells compared with solid tumour cell lines, liver metastases and normal liver.

van der Wilt CL, Kroep JR, Loves WJ, Rots MG, Van Groeningen CJ, Kaspers GJ, Peters GJ.

Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands.

Deoxycytidine kinase (dCK) is required for the phosphorylation of several deoxyribonucleoside analogues that are widely employed as chemotherapeutic agents. Examples include cytosine arabinoside (Ara-C) and 2-chlorodeoxyadenosine (CdA) in the treatment of acute myeloid leukaemia (AML) and gemcitabine to treat solid tumours. In this study, expression of dCK mRNA was measured by a competitive template reverse transcriptase polymerase chain reaction (CT RT-PCR) in seven cell lines of different histological origin, 16 childhood and adult AML samples, 10 human liver samples and 11 human liver metastases of colorectal cancer origin. The enzyme activity and protein expression levels of dCK in the cell lines were closely related to the mRNA expression levels (r=0.75, P=0.026 and r=0.86, P=0.007). In AML samples, dCK mRNA expression ranged from 1.16 to 35.25 (x10(-3)xdCK/beta-actin). In the cell line panel, the range was 2.97-56.9 (x10(-3)xdCK/beta-actin) of dCK mRNA expression. The enzyme activity in liver metastases was correlated to dCK mRNA expression (r=0.497, P=0.05). In the liver samples, these were not correlated. dCK mRNA expression showed only a 36-fold range in liver while a 150-fold range was observed in the liver metastases. In addition, dCK activity and mean mRNA levels were 2.5-fold higher in the metastases than in the liver samples. Since dCK is associated with the sensitivity to deoxynucleoside analogues and because of the good correlation between the different dCK measurements in malignant cells and tumours, the CT-RT PCR assay will be useful in the selection of patients that can be treated with deoxycytidine analogues.

PMID: 12628850 [PubMed - indexed for MEDLINE]

EXHIBIT 17

67: Regul Pept. 2004 Feb 15;117(2):127-39. Related Articles, Links

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Galanin in pituitary adenomas.

Grenback E, Bjellerup P, Wallerman E, Lundblad L, Anggard A, Ericson K, Aman K, Landry M, Schmidt WE, Hokfelt T, Hulting AL.

Department of Molecular Medicine, Endocrine and Diabetes Unit, Karolinska Hospital, S-17176 Stockholm, Sweden. Eva.Grenback@ks.se

Tumor galanin content was measured in extracts from human pituitary adenomas using a specific RIA method for monitoring human galanin. Twenty-two out of twenty-four tumors contained galanin with notably high levels in corticotroph adenomas, varying levels in clinically inactive tumors, and low levels in GH secreting adenomas. Tumor galanin and ACTH contents were closely correlated in all tumors. In four young patients with microadenomas and highly active Mb Cushing tumor galanin was inversely related to tumor volume. The molecular form of tumor galanin, studied with reverse-phase HPLC, was homogeneous with the majority of tumor galanin coeluting with standard human galanin. In the tumors analysed with in situ hybridization there was a good correlation between galanin peptide levels and galanin mRNA expression. In some tumors galanin mRNA and POMC levels coexisted, in others they were essentially in different cell populations. Levels of plasma galanin-LI were not related to tumor galanin concentration, and galanin levels were in the same range in sinus petrosus close to the pituitary venous drainage as in peripheral blood. Corticotrophin releasing hormone injections in two patients caused ACTH, but no detectable galanin release into sinus petrosus. Our results demonstrate that corticotroph, but not GH adenomas, express high levels of galanin, in addition to ACTH, and that in some tumors both polypeptides are synthesised in the same cell population. However, galanin levels in plasma were not influenced by the tumor galanin content.

PMID: 14700749 [PubMed - indexed for MEDLINE]

EXHIBIT 18

50: Blood. 2004 Nov 1;104(9):2936-9. Epub 2004 Jul 8.

BCL2 protein expression parallels its mRNA level in normal and malignant B cells.

Shen Y, Iqbal J, Huang JZ, Zhou G, Chan WC.

Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, USA.

The regulation of B-cell lymphoma 2 (BCL2) protein expression in germinal center (GC) B cells has been controversial. Previous reports have indicated posttranscriptional regulation plays a dominant role. However, a number of recent studies contradicted these reports. Using real-time polymerase chain reaction (PCR) and Standardized Reverse Transcriptase-PCR (StaRT-PCR), we measured the level of mRNA expression in GC, mantle zone (MNZ), and marginal zone (MGZ) cells from laser capture microdissection. Both quantitative RT-PCR measurements of microdissected GC cells from tonsils showed that GC cells had low expression of BCL2 transcripts commensurate with the low protein expression level. These results are in agreement with microarray studies on fluorescence-activated cell sorter (FACS)-sorted cells and microdissected GC cells. We also examined BCL2 mRNA and protein expression on a series of 30 cases of diffuse large B-cell lymphoma (DLBCL) and found, in general, a good correlation. The results suggested that BCL2 protein expression is regulated at the transcriptional level in normal B cells and in the neoplastic cells in most B-cell lymphoproliferative disorders.

PMID: 15242877 [PubMed - indexed for MEDLINE]

EXHIBIT 19

1: Blood. 2005 Dec 15;106(13):4315-21. Epub 2005 Aug 25. Related Articles, Links

Full text article at www.bloodjournal.org

Cyclin D1-negative mantle cell lymphoma: a clinicopathologic study based on gene expression profiling.

Fu K, Weisenburger DD, Greiner TC, Dave S, Wright G, Rosenwald A, Chiorazzi M, Iqbal J, Gesk S, Siebert R, De Jong D, Jaffe ES, Wilson WH, Delabie J, Ott G, Dave BJ, Sanger WG, Smith LM, Rimsza L, Braziel RM, Muller-Hermelink HK, Campo E, Gascoyne RD, Staudt LM, Chan WC; Lymphoma/Leukemia Molecular Profiling Project.

Department of Pathology and Microbiology, University of Nebraska Medical Center, 983135 Nebraska Medical Center, Omaha, NE 68198-3135, USA. kfu@unmc.edu

Cyclin D1 overexpression is believed to be essential in the pathogenesis of mantle cell lymphoma (MCL). Hence, the existence of cyclin D1-negative MCL has been controversial and difficult to substantiate. Our previous gene expression profiling study identified several cases that lacked cyclin D1 expression, but had a gene expression signature typical of MCL. Herein, we report the clinical, pathologic, and genetic features of 6 cases of cyclin D1-negative MCL. All 6 cases exhibited the characteristic morphologic features and the unique gene expression signature of MCL but lacked the t(11;14)(q13; q32) by fluorescence in situ hybridization (FISH) analysis. The tumor cells also failed to express cyclin D1 protein, but instead expressed either cyclin D2 (2 cases) or cyclin D3 (4 cases). There was good correlation between cyclin D protein expression and the corresponding mRNA expression levels by gene expression analysis. Using interphase FISH, we did not detect chromosomal translocations or amplifications involving CCND2 and CCND3 loci in these cases. Patients with cyclin D1-negative MCL were similar clinically to those with cyclin D1-positive MCL. In conclusion, cases of cyclin D1-negative MCL do exist and are part of the spectrum of MCL. Up-regulation of cyclin D2 or D3 may substitute for cyclin D1 in the pathogenesis of MCL.

PMID: 16123218 [PubMed - indexed for MEDLINE]

EXHIBIT 20

3: J Gen Virol. 2005 Oct;86(Pt 10):2769-80. Related Articles, Links

The alpha(v)beta6 integrin receptor for Foot-and-mouth disease virus is expressed constitutively on the epithelial cells targeted in cattle.

Monaghan P, Gold S, Simpson J, Zhang Z, Weinreb PH, Violette SM, Alexandersen S, Jackson T.

Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Surrey GU24 0NF, UK.

Field strains of Foot-and-mouth disease virus (FMDV) use a number of alpha(v)-integrins as receptors to initiate infection on cultured cells, and integrins are believed to be the receptors used to target epithelial cells in animals. In this study, immunofluorescence confocal microscopy and real-time RT-PCR were used to investigate expression of two of the integrin receptors of FMDV, alpha(v)beta6 and alpha(v)beta3, within various epithelia targeted by this virus in cattle. These studies show that alpha(v)beta6 is expressed constitutively on the surfaces of epithelial cells at sites where infectious lesions occur during a natural infection, but not at sites where lesions are not normally formed. Expression of alpha(v)beta6 protein at these sites showed a good correlation with the relative abundance of beta6 mRNA. In contrast, alpha(v)beta3 protein was only detected at low levels on the vasculature and not on the epithelial cells of any of the tissues investigated. Together, these data suggest that in cattle, alpha(v)beta6, rather than alpha(v)beta3, serves as the major receptor that determines the tropism of FMDV for the epithelia normally targeted by this virus.

PMID: 16186231 [PubMed - in process]

EXHIBIT 21

Neuroscience. 2005;136(1):147-60. Epub 2005 Sep 21.

Modulation of the glutamatergic receptors (AMPA and NMDA) and of glutamate vesicular transporter 2 in the rat facial nucleus after axotomy.

Eleore L, Vassias I, Vidal PP, de Waele C.

LNRS (CNRS-Paris V), ESA 7060, Centre Universitaire des Saints-Peres, 45 rue des Saints-Peres, 75270 Paris Cedex 06, France.

Facial nerve axotomy is a good model for studying neuronal plasticity and regeneration in the peripheral nervous system. We investigated in the rat the effect of axotomy on the different subunits of excitatory glutamatergic AMPA (GLuR1-4), NMDA (NR1, NR2A-D) receptors, post-synaptic density 95, vesicular glutamate transporter 2, beta catenin and cadherin. mRNA levels and/or protein production were analyzed 1, 3, 8, 30 and 60 days after facial nerve axotomy by in situ hybridization and immunohistofluorescence. mRNAs coding for the GLuR2-4, NR1, NR2A, B, D subunits of glutamatergic receptors and for post-synaptic density 95, were less abundant after axotomy. The decrease began as early as 1 or 3 days after axotomy; the mRNAs levels were lowest 8 days post-lesion, and returned to normal or near normal 60 days after the lesion. The NR2C subunit mRNAs were not detected in either lesioned or intact facial nuclei. Immunohistochemistry using specific antibodies against GLuR2-3 subunits and against NR1 confirmed this down-regulation. There was also a large decrease in vesicular glutamate transporter 2 immunostaining in the axotomized facial nuclei at early stages following facial nerve section. In contrast, no decrease of NR2A subunit and of post-synaptic density 95 could be detected at any time following the lesion. beta Catenin and cadherin immunoreactivity pattern changed around the cell body of facial motoneuron by day 3 after axotomy, and then, tends to recover at day post-lesion 60 days. Therefore, our results suggest a high correlation between restoration of nerve/muscle synaptic contact, synaptic structure and function in facial nuclei. To investigate the mechanisms involved in the change of expression of these proteins following axotomy, the facial nerve was perfused with tetrodotoxin for 8 days. The blockade of action potential significantly decreased GLuR2-3, NR1and NR2A mRNAs in the ipsilateral facial nuclei. Thus, axotomy-induced changes in mRNA abundance seemed to depend partly on disruption of activity.

PMID: 16182453 [PubMed - in process]

EXHIBIT 22

SECOND DECLARATION OF PAUL POLAKIS, Ph.D.

I, Paul Polakis, Ph.D., declare and say as follows:

1. I am currently employed by Genentech, Inc. where my job title is Staff Scientist.

2. Since joining Genentech in 1999, one of my primary responsibilities has been leading Genentech's Tumor Antigen Project, which is a large research project with a primary focus on identifying tumor cell markers that find use as targets for both the diagnosis and treatment of cancer in humans.

3. As I stated in my previous Declaration dated May 7, 2004 (attached as Exhibit A), my laboratory has been employing a variety of techniques, including microarray analysis, to identify genes which are differentially expressed in human tumor tissue relative to normal human tissue. The primary purpose of this research is to identify proteins that are abundantly expressed on certain human tumor tissue(s) and that are either (i) not expressed, or (ii) expressed at detectably lower levels, on normal tissue(s).

4. In the course of our research using microarray analysis, we have identified approximately 200 gene transcripts that are present in human tumor tissue at significantly higher levels than in normal human tissue. To date, we have successfully generated antibodies that bind to 31 of the tumor antigen proteins expressed from these differentially expressed gene transcripts and have used these antibodies to quantitatively determine the level of production of these tumor antigen proteins in both human tumor tissue and normal tissue. We have then quantitatively compared the levels of mRNA and protein in both the tumor and normal tissues analyzed. The results of these analyses are attached herewith as Exhibit B. In Exhibit B, "+" means that the mRNA or protein was detectably overexpressed in the tumor tissue relative to normal tissue and "-" means that no detectable overexpression was observed in the tumor tissue relative to normal tissue.

5. As shown in Exhibit B, of the 31 genes identified as being detectably overexpressed in human tumor tissue as compared to normal human tissue at the mRNA level, 28 of them (i.e., greater than 90%) are also detectably overexpressed in human tumor tissue as compared to normal human tissue at the protein level. As such, in the cases where we have been able to quantitatively measure both (i) mRNA and (ii) protein levels in both (i) tumor tissue and (ii) normal tissue, we have observed that in the vast majority of cases, there is a very strong correlation between increases in mRNA expression and increases in the level of protein encoded by that mRNA.

6. Based upon my own experience accumulated in more than 20 years of research, including the data discussed in paragraphs 4-5 above and my knowledge of the relevant scientific literature, it is my considered scientific opinion that for human genes, an increased level of mRNA in a tumor tissue relative to a normal tissue more often than not correlates to a similar increase in abundance of the encoded protein in the tumor tissue relative to the normal tissue. In fact, it remains a generally accepted working assumption in molecular biology that increased mRNA levels are more often than not predictive of elevated levels of the encoded protein. In fact, an entire industry focusing on the research and development of therapeutic antibodies to treat a variety of human diseases, such as cancer, operates on this working assumption.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 3-29-06

By: Paul Polakis

Paul Polakis, Ph.D.

EXHIBIT A

DECLARATION OF PAUL POLAKIS, Ph.D.

I, Paul Polakis, Ph.D., declare and say as follows:

1. I was awarded a Ph.D. by the Department of Biochemistry of the Michigan State University in 1984. My scientific Curriculum Vitae is attached to and forms part of this Declaration (Exhibit A).

2. I am currently employed by Genentech, Inc. where my job title is Staff Scientist. Since joining Genentech in 1999, one of my primary responsibilities has been leading Genentech's Tumor Antigen Project, which is a large research project with a primary focus on identifying tumor cell markers that find use as targets for both the diagnosis and treatment of cancer in humans.

3. As part of the Tumor Antigen Project, my laboratory has been analyzing differential expression of various genes in tumor cells relative to normal cells. The purpose of this research is to identify proteins that are abundantly expressed on certain tumor cells and that are either (i) not expressed, or (ii) expressed at lower levels, on corresponding normal cells. We call such differentially expressed proteins "tumor antigen proteins". When such a tumor antigen protein is identified, one can produce an antibody that recognizes and binds to that protein. Such an antibody finds use in the diagnosis of human cancer and may ultimately serve as an effective therapeutic in the treatment of human cancer.

4. In the course of the research conducted by Genentech's Tumor Antigen Project, we have employed a variety of scientific techniques for detecting and studying differential gene expression in human tumor cells relative to normal cells, at genomic DNA, mRNA and protein levels. An important example of one such technique is the well known and widely used technique of microarray analysis which has proven to be extremely useful for the identification of mRNA molecules that are differentially expressed in one tissue or cell type relative to another. In the course of our research using microarray analysis, we have identified approximately 200 gene transcripts that are present in human tumor cells at significantly higher levels than in corresponding normal human cells. To date, we have generated antibodies that bind to about 30 of the tumor antigen proteins expressed from these differentially expressed gene transcripts and have used these antibodies to quantitatively determine the level of production of these tumor antigen proteins in both human cancer cells and corresponding normal cells. We have then compared the levels of mRNA and protein in both the tumor and normal cells analyzed.

5. From the mRNA and protein expression analyses described in paragraph 4 above, we have observed that there is a strong correlation between changes in the level of mRNA present in any particular cell type and the level of protein

expressed from that mRNA in that cell type. In approximately 80% of our observations we have found that increases in the level of a particular mRNA correlates with changes in the level of protein expressed from that mRNA when human tumor cells are compared with their corresponding normal cells.

6. Based upon my own experience accumulated in more than 20 years of research, including the data discussed in paragraphs 4 and 5 above and my knowledge of the relevant scientific literature, it is my considered scientific opinion that for human genes, an increased level of mRNA in a tumor cell relative to a normal cell typically correlates to a similar increase in abundance of the encoded protein in the tumor cell relative to the normal cell. In fact, it remains a central dogma in molecular biology that increased mRNA levels are predictive of corresponding increased levels of the encoded protein. While there have been published reports of genes for which such a correlation does not exist, it is my opinion that such reports are exceptions to the commonly understood general rule that increased mRNA levels are predictive of corresponding increased levels of the encoded protein.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information or belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardize the validity of the application or any patent issued thereon.

Dated: 5/07/04

By: Paul Polakis

Paul Polakis, Ph.D.

SV 2031808 v1

CURRICULUM VITAE

PAUL G. POLAKIS
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EDUCATION:

Ph.D., Biochemistry, Department of Biochemistry,
Michigan State University (1984)

B.S., Biology. College of Natural Science, Michigan State University (1977)

PROFESSIONAL EXPERIENCE:

2002-present	Staff Scientist, Genentech, Inc S. San Francisco, CA
1999- 2002	Senior Scientist, Genentech, Inc., S. San Francisco, CA
1997 -1999	Research Director Onyx Pharmaceuticals, Richmond, CA
1992- 1996	Senior Scientist, Project Leader, Onyx Pharmaceuticals, Richmond, CA
1991-1992	Senior Scientist, Chiron Corporation, Emeryville, CA.
1989-1991	Scientist, Cetus Corporation, Emeryville CA.
1987-1989	Postdoctoral Research Associate, Genentech, Inc., South SanFrancisco, CA.
1985-1987	Postdoctoral Research Associate, Department of Medicine, Duke University Medical Center, Durham, NC

1984-1985	Assistant Professor, Department of Chemistry, Oberlin College, Oberlin, Ohio
1980-1984	Graduate Research Assistant, Department of Biochemistry, Michigan State University East Lansing, Michigan

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EXHIBIT B

	tumor mRNA	tumor IHC
UNQ2525	+	+
UNQ2378	+	+
UNQ972	+	-
UNQ97671	+	+
UNQ2964	+	+
UNQ323	+	+
UNQ1655	+	+
UNQ2333	+	+
UNQ9638	+	+
UNQ8209	+	+
UNQ6507	+	+
UNQ8196	+	+
UNQ9109	+	+
UNQ100	+	+
UNQ178	+	+
UNQ1477	+	+
UNQ1839	+	+
UNQ2079	+	+
UNQ8782	+	+
UNQ9646	+	-
UNQ111	+	+
UNQ3079	+	+
UNQ8175	+	+
UNQ9509	+	+
UNQ10978	+	-
UNQ2103	+	+
UNQ1563	+	+
UNQ16188	+	+
UNQ13589	+	+
UNQ1078	+	+
UNQ879	+	+